

FOCH1

PTO/PCT Rec'd 27 JUL 2000

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

09/601138

INTERNATIONAL APPLICATION NO.  
PCT/DK99/00040

INTERNATIONAL FILING DATE  
27 JANUARY 1999

PRIORITY CLAIMED  
27 JANUARY 1998

## TITLE OF INVENTION

METHOD FOR TREATING ACUTE INTERMITTENT PORPHYRIA (AIP) AND OTHER ...

## APPLICANT(S) FOR DO/EO/US

Jens FOGH et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
  - ☒ Courtesy copy of the International Application as filed.
  - ☒ Courtesy copy of the first page of the International Publication (WO 99/37325).
  - ☒ Courtesy copy of the International Preliminary Examination Report with annexes containing Figures 3, 4 and 6 to be substituted for original Figures 3, 4 and 6 for examination in this case.
  - ☒ Formal drawings, 31 sheets, Figures 1-9x.
  - ☒ Courtesy copy of the International Search Report.
  - ☒ Paper copy of Sequence Listing.

**09/601138****PCT/DK99/00040****FOGH1**17. **[XX]** The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):**

Neither international preliminary examination fee (37 CFR 1.482)

nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO.....\$970.00International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO.....\$840.00International preliminary examination fee (37 CFR 1.482) not paid to USPTO but  
international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$760.00International preliminary examination fee paid to USPTO (37 CFR 1.482)  
but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$670.00International preliminary examination fee paid to USPTO (37 CFR 1.482)  
and all claims satisfied provisions of PCT Article 33(1)-(4).....\$96.00**ENTER APPROPRIATE BASIC FEE AMOUNT =**Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

Claims as Originally Presented	Number Filed	Number Extra	Rate
Total Claims	44 - 20	24	X \$18.00
Independent Claims	4 - 3	1	X \$78.00
Multiple Dependent Claims (if applicable)			+\$260.00

**TOTAL OF ABOVE CALCULATIONS =**

Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate
Total Claims	- 20		X \$18.00
Independent Claims	- 3		X \$78.00

**TOTAL OF ABOVE CALCULATIONS =**Reduction of ½ for filing by small entity, if applicable. A small entity statement  
must also be filed (Note 37 CFR 1.9, 1.27, 1.28)**SUBTOTAL =**Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30  
months from the earliest claimed priority date (37 CFR 1.492(f)).**TOTAL NATIONAL FEE =**Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +**TOTAL FEES ENCLOSED =****CALCULATIONS PTO USE ONLY**

\$ 840.00

\$

\$ 432.00

\$ 78.00

\$

\$ 1,350.00

\$ 1,350.00

\$ 1,350.00

\$ 1,350.00

\$

\$ 1,350.00

Amount to be: \$

refunded

charged \$

a. [ ] A check in the amount of \$ to cover the above fees is enclosed.

b. **[XX]** Credit Card Payment Form (PTO-2038), authorizing payment in the amount of **\$1,350.00**, is attached.c. [ ] Please charge my Deposit Account No. **02-4035** in the amount of \$ to cover the above fees.

A duplicate copy of this sheet is enclosed.

d. **[XX]** The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment  
to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or  
(b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**BROWDY AND NEIMARK, P.L.L.C.**  
**624 NINTH STREET, N.W., SUITE 300**  
**WASHINGTON, D.C. 20001**

TEL: (202) 628-5197

FAX: (202) 737-3528

Date of this submission: **July 27, 2000**

SIGNATURE

Iver P. Cooper

NAME

28,005

REGISTRATION NUMBER

Applicant or Patentee: Par Gellerfors et al. Case Docket No. \_\_\_\_\_  
Serial or Patent No.: \_\_\_\_\_  
Filed or Issued: \_\_\_\_\_  
or: Method for treating acute intermittent porphyria (AIP) and other  
porphyric diseases

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

- ☐ the owner of the small business concern identified below.  
☒ an official of the small business concern empowered to act on behalf of the concern identified below.

NAME OF CONCERN Hemebiotech A/S  
ADDRESS OF CONCERN Roskildevej 12 C, DK-3400 Hillerød, Denmark

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled Method for treating acute intermittent porphyria (AIP) and other porphyric diseases by inventor (s) Par Gellerfors and Jens Fogh described in \_\_\_\_\_

- ☐ the specification filed herewith  
☒ application serial no. \_\_\_\_\_, filed 27 July 2000  
☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

If the right held by the above identified small business concern is not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate.  
(37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING JENS FOGH Par Gellerfors  
TITLE OF PERSON OTHER THAN OWNER \_\_\_\_\_  
ADDRESS OF PERSON SIGNING HEMEBIOTECH A/S ROSKILDEVEJ 12 C  
DK-3400 HILLERØD  
SIGNATURE [Signature] DATE 24 August 2000

534 Rec'd PCT/PTC 27 JUL2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
Jens FOGH et al.	)	
	)	
IA No.: PCT/DK99/00040	)	
	)	Washington, D.C.
IA Filed: January 27, 1999	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	July 27, 2000
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: METHOD FOR TREATING...	)	Docket No.: FOGH1

PRELIMINARY AMENDMENT

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE CLAIMS

The claims set as filed includes two claims numbered "36". In accordance with U.S. Practice, the second claim 36 should be numbered "37", and 37-45 should be renumbered as 38-46 (with appropriate renumbering of dependencies in claims referring to claims original numbered 37-45).

The amendments set forth below refer to the claims as originally numbered.

In claim 3, delete "or 2".

In claims 4-6, 16-17, 26, 28-31, replace "any of the preceding claims" with --claim 1--.

In claims 7-13, 15, 20, 22, 27, 35, 36, 40-42, replace "any of claims with --claim--.

In claim 24, replace "claims" with --claim--.

In the claims indicated below, delete the indicated

text

CLAIM	TEXT
7	-6
8	-7
9	-8
10-12	-6, 8 or 9
13, 15, 22	-9
24	-19
27	-25
35, 36	-31
40	-39
41	-40
42	-41

In claim 45, delete "or 44".

Please cancel claims 33-34.

In claim 32, at the beginning of the claim, insert --A pharmaceutical composition comprising--, and at the end delete ", for use as a medicament".

In claim 16, delete "such as ... microspheres".

In claim 29, delete "such as 2, 3, 4 and 5 times daily".

In claim 30, delete "such as ... day".

In claim 7, delete "such as ... salts".

In claim 12, delete "such as ... these", and replace "involving" with --comprising--.

In claim 43, line 2, replace "by a" with --comprising--.

Please rewrite claim 44 as follows:

44 (Amended). The method according to [any of claims] claim 43 wherein said correction is effected by transfection with a delivery system which delivers a corrected gene, and wherein the delivery system for transfection is by use of non-viral vectors formulated in a vehicle preparation comprising one or more components selected from cationic phospholipids, phospholipids, phospholipids mixed with neutral lipids, lictosylated PEI, liposomes liposomes comprising mixtures of natural phospholipids and neutral lipids.

In claim 41, line 2, after "selected from" insert --the group consisting of--.

If, inadvertently, a proper multiple dependent claim has not been amended to reduce it to single dependency, please amend it to be dependent solely on the first-mentioned claim, or, if that is not possible, please cancel the claim and notify the undersigned.

#### REMARKS

The above amendments to the claims are being made in order to eliminate any properly multiply dependent claims, for the purpose of reducing the filing fee and also to place the application in better condition for examination. Please enter this amendment prior to calculation of the filing fee in this case.

Respectfully submitted,  
BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By:

Iver P. Cooper

Registration No. 37,971

IPC:edg

Telephone No.: (202) 628-5197

Facsimile No.: (202) 737-3528

F:\,P\Plou\fogh1\pto\preliminary amendment.doc

METHOD FOR TREATING ACUTE INTERMITTENT PORPHYRIA (AIP) AND OTHER PORPHYRIC DISEASES**5 FIELD OF THE INVENTION**

The present invention relates to novel methods of treating and preventing disease caused by absence or deficiency of the activity of enzymes belonging to the heme biosynthetic pathway. More specifically, the invention pertains to methods of  
10 alleviating the symptoms of certain porphyrias, notably acute intermittent porphyria including gene therapy.

**BACKGROUND OF THE INVENTION****15 Heme biosynthetic pathway**

Heme is a vital molecule for life in all living higher animal species. Heme is involved in such important processes as oxygen transportation (haemoglobin), drug detoxification (cytochrome P450), and electron transfer for the generation of chemical energy (ATP)  
20 during oxidative phosphorylation in mitochondria.

Heme is synthesised in eight consecutive enzymatic steps starting with glycine and succinyl-CoA. Sassa S. 1996, Blood Review, 10, 53-58 shows a schematic drawing of the heme biosynthetic pathway indicating that that the first enzymatic step (ALA-  
25 synthetase) and the last three steps (coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase) are located in the mitochondrion whereas, the remaining are cytosolic enzymes.

Important regulation of the heme biosynthetic pathway is delivered by the end product  
30 of the metabolic pathway, namely heme, which exerts a negative inhibition on the first rate-limiting enzymatic step (conducted by ALA-synthetase) in the heme biosynthetic pathway. (Strand et al. Proc. Natl. Acad. Sci. 1970, 67, 1315-1320).



Deficiencies in the heme biosynthetic enzymes have been reported leading to a group of diseases collectively called porphyrias.

A defect in the third enzymatic step leads to acute intermittent porphyria, AIP.

5

#### Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is an autosomal dominant disorder in man caused by a defect (50% reduction of activity) of the third enzyme in the heme biosynthetic pathway, porphobilinogen deaminase, (also known as porphobilinogen ammonia-  
10 lyase (polymerizing)), E.C. 4.3.1.8. (Waldenström J. Acta. Med. Scand. 1937 Suppl. 82). In the following, this enzyme will be termed "PBGD".

#### Clinical manifestation of AIP

15

The reduction in enzymatic PBGD activity makes this enzyme the rate limiting step in the heme biosynthetic pathway, with a concomitant increase in urinary and serum levels of delta-aminolevulinic acid (ALA) and porphobilinogen (PBG).

20 The clinical manifestation of AIP involves abdominal pain and a variety of neuropsychiatric and circulatory dysfunctions. As a result of the enzymatic block, heme precursors such as porphobilinogen (PBG) and delta-aminolevulinic acid (ALA) are excreted in excess amounts in the urine and stool. In acute attacks, high levels of PBG and ALA are also found in serum. These precursors are normally undetectable in  
25 serum in healthy individuals.

The neuropsychiatric disturbances observed in these patients are thought to be due to interference of the precursors with the nervous system or due to the lack of heme. For instance, ALA bears a close resemblance to the inhibitory neurotransmitter 4-  
30 aminobutyric acid (GABA) and has been suggested to be a neurotoxin. (Jeans J. et al. American J. of Medical Genetics 1996, 65, 269-273).

Abdominal pain is the most frequent symptom in AIP patients and occurs in more than 90% during acute attacks, which will be followed rapidly by the development of

peripheral neuropathy with weakness in proximal muscles, loss of pinprick sensation, and paraesthesia. Tachycardia, obstipation or diarrhoea may also be present.

During acute attacks behavioural changes, confusion, seizures, respiratory paralysis, coma and hallucinations may be present.

5

Hypertension is also associated with AIP, with as high as 40% of patients showing sustained hypertension between attacks. An association between chronic renal failure (Yeung L. et al. 1983, Q J. Med 52, 92-98) and AIP as well as hepatocellular carcinoma. (Lithner F. et al. 1984, Acta.Med.Scand. 215,271-274), has been

10 reported.

The AIP is a lifelong disease, which usually becomes manifest in puberty.

#### Factors precipitating acute attacks.

15

Most precipitating factors exhibit an association with the first rate-limiting enzyme in the heme biosynthetic pathway through heme, the final product of the pathway. A lowering of the heme concentration will immediately increase the rate of ALA-synthetase. An overproduction of ALA then makes the partially deficient PBGD enzyme (50% activity) now rate-limiting with an accumulation of the heme precursors ALA and PBG. Drugs that induces cytochrome P450 such as barbiturates, estrogens, sulphonamides, progesterone, carbamazepine, and phenytoin can all precipitate acute attacks. (Wetterberg L. 1976, In Doss M. Nowrocki P. eds. Porphyrias in Human Disease. Reports of the discussion. Matgurg an der Lahn, 191-202).

25

The clinical manifestation is more common in women, especially at time of menstruation. Endocrine factors such as synthetic estrogens and progesterone are known precipitating factors. A significant factor is also the lack of sufficient caloric intake. Hence, caloric supplementation during acute attacks reduces clinical symptoms. (Welland F.H. et al. 1964, Metabolism,13, 232).

30

Finally, various forms of stress including illness, infections, surgery and alcoholic excess have been shown to lead to precipitation of acute attacks. There are also cases of acute attacks where no precipitating factor can be identified.

### Prevalence of AIP

Prevalence of 0.21% has been reported (Tishler P.V: et al. 1985, Am.J.Psychiatry 142,1430-1436), with as high a prevalence as 1 per 1500 in geographic isolates in northern Sweden (Wetterberg L. 1967, Svenska bokförlaget Nordstedt, Stockholm). Prevalence up to 200 per 10,000 inhabitants have been reported from Arjepung in Northern Sweden. (Andersson, Christer, Thesis, 1997, ISBN 91/7191/280/0, pp. 22-23).

### 10 Existing treatment of AIP

The treatment of AIP as well as of other types of porphyrias such as variegata, hereditary coproporphyria, harderoporphyria, and aminolevulinic acid dehydratase deficiency, are basically the same. Existing therapies for AIP, are all aimed at reducing circulating PBG and ALA by inhibiting the first rate-limiting enzymatic step ALA-synthetase. This inhibition of ALA-synthetase is achieved by increasing circulating heme, since heme is a negative feed back regulator of ALA-synthetase. Hematin treatment, high caloric intake or inhibition of heme breakdown by Sn-mesoporphyrin administration are the existing therapies today. These therapies have shown limited efficacy.

Treatment between acute attacks involves sufficient caloric intake and avoidance of drugs and immediate treatment of infections.

25 Patients that experience acute attacks are treated with intravenous carbohydrates usually dextrose (300 g/day) and intravenous hematin (3 - 8 mg/(kg day)).

Treatments with long acting agonistic analogues of LHRH, have been shown to reduce the incidence of pre-menstrual attacks by inhibiting ovulation in AIP patients. Finally,

30 treatments involving heme analogues Sn-mesoporphyrin, which inhibit heme breakdown have also been attempted.

### Medical need in AIP

The lack of effective treatment for AIP is well recognised. In a US mortality study in AIP patients requiring hospitalisation it was concluded that the mortality rate was 3.2-5 fold higher as compared to a matched general population.

Suicide was also a major cause of death, occurring at a rate of 370 times that expected in the general population (Jeans J. et al. 1996, Am. J. of Medical Genetics 65, 269-273).

10

Hematin therapy is usually initiated when high caloric intake is not sufficient to alleviate acute attacks. Studies with hematin have been performed but these studies generally used the patients as their own control after the patients did not respond to high carbohydrate treatment. (Mustajoki et al. 1989, Sem. Hematol. 26, 1-9).

15

The one controlled study with hematin treatment reported, failed to reach statistical significance due to too small a patient number (Herrick A.L. et al 1989, Lancet 1, 1295-1297).

20 In conclusion, there is a definite need for the provision of novel therapeutic/prophylactic methods aimed at these diseases.

### **DISCLOSURE OF THE INVENTION**

25 Levels of ALA and PBG found in urine in patients with symptomatic AIP, are in the range of 1-203 mg/day and 4-782 mg/day, respectively. Normal excretion of ALA and PBG is very low (0-4 mg/day). Important is the observation that these patients also have elevated levels of ALA and PBG in serum. It was shown in a study that AIP patients had significantly elevated levels of ALA (96  $\mu$ g %) and PBG (334  $\mu$ g %) in  
30 serum in connection with acute attacks and that the severity of the attacks were correlated to high levels of ALA and PBG. Hence, it is important to reduce the circulating levels of ALA and PBG in order to eliminate clinical symptoms and to normalize the heme pool.

The present inventors present a new therapeutic rational in the treatment of AIP, a rationale using PBGD, preferably recombinant PBGD (rPBGD) in order to reduce circulating high levels of PBG in serum by metabolising (by enzymatic conversion) PBG to hydroxymethylbilane (HMB), which is the normal product of the reaction. This

- 5 substitution therapy will lead to a normalization of PBG in serum as well as to a normalization of the heme pool. It will also lead to a normalization of ALA in serum, since these heme precursors are in equilibrium with each other. A lowering of serum ALA and PBG is expected to result in a concomitant relief of symptoms. The product of the reaction (HMB) will diffuse back into the cells and enter the normal heme
- 10 biosynthetic pathway and will become subsequently metabolized to heme.

- Hence, PBGD administered by injections will carry out its normal catalytic function by converting PBG to HMB in serum (extracellularly, not inside the cells), where it normally functions. The new therapeutic idea is based on the assumption that ALA,
- 15 PBG and HMB permeate cellular membranes or are transported specifically across them. An alternative to this is to administer a form of PBGD which will be able to act intracellularly, either as a consequence of formulation or as consequence of modification of PBGD so as to facilitate its entry into cells from the extracellular compartment.

20

The observation that AIP patients have large amounts of these heme precursors in the serum supports the idea that PBG does not accumulate intracellularly, but is released from the cells into serum when the intracellular concentration increases due to the PBGD enzymatic block.

25

The basic new therapeutic concept for AIP is valid for all porphyrias and therefore the invention is in general aimed at treating these diseases by substituting the reduced or missing enzymatic activity characterizing the porphyrias.

- 30 Hence, in its broadest aspect, the invention pertains to a method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof.

Alternatively investigations in treating the porphyrias have also suggested gene therapy, thus aiming at introducing genetic material in relevant cells, which will then take over the *in vivo* production of the enzyme of interest.

Hence, by the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or subsequence of the enzyme which includes the necessary catalytic site to enable the domain or subsequence to exert substantially the same enzymatic activity as the full-length enzyme or alternatively a gene coding for the catalyst.

An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely synthetic molecules which mimic the specific enzymatic activity of the relevant enzyme would also constitute "enzymatic equivalent analogues".

In essence, the inventive concept is based on the novel idea of substituting the reduced enzymatic activity in the subject simply by administering a catalyst which will "assist" the enzyme which is in deficit. The precise nature, however, of the catalyst is not all-important. What is important is merely that the catalyst can mimic the enzymatic *in vivo* activity of the enzyme.

The term "the heme biosynthetic pathway" refers to the well-known enzymatic steps (cf. e.g. Sassa S. 1996, Blood Review, 10, 53-58) which leads from glycine and succinyl-CoA to heme, and enzymes belonging to this synthetic pathway are uroporphobilinogen deaminase (PBGD), ALA dehydratase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, coproporphyrinogen oxidase, protoporphyrinogen oxidase, uroporphyrinogen III synthase, ferrochelatase, and uroporphyrinogen decarboxylase. Hence, in line with the above, a catalyst used according to the invention is such an enzyme or an enzymatically equivalent part or analogue thereof. It should be noted that all of the above-mentioned enzymes have been sequenced, thus allowing recombinant or synthetic production thereof.

The diseases related to reduced activity of these enzymes are acute intermittent porphyria (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP),

5 Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

By the term "effective amount" is herein meant a dosage of the catalyst which will supplement the lack or deficiency of enzymatic activity in a subject suffering from

10 porphyria caused by reduced activity of one of the above-mentioned enzymes. The precise dosage constituting an effective amount will depend on a number of factors such as serum half-life of the catalyst, specific activity of the catalyst etc. but the skilled person will be able to determine the correct dosage in a given case by means of standard methods (for instance starting out with experiments in a suitable animal

15 model such as with transgenic animals so as to determine the correlation between blood concentration and enzymatic activity).

The disease which is the preferred target for the inventive method is AIP, and therefore the catalyst is PBGD or an enzymatically equivalent part or analogue thereof.

20 It is most preferred that the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof, since recombinant production will allow large-scale production which, with the present means available, does not seem feasible if the enzyme would have to be purified from a native source.

25

Preferred formulations and dosage forms of the catalyst are exemplified for, but not limited to, PBGD in the detailed description hereinafter, and these formulations also are apparent from the claims. It will be appreciated that these formulations and dosage forms are applicable for all catalysts used according to the invention.

30

One important embodiment of the method of the inventions is one wherein the catalyst, upon administration, exerts at least part of its enzymatic activity in the intracellular compartment. This can e.g. be achieved when the catalyst is an enzymatically equivalent part or analogue of the enzyme, since such variations of the

enzyme can be tailored to render them permeate cell membranes. Hence, when the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane, it should be possible for the skilled man to introduce relevant side chains which facilitates entry into the intracellular compartment. Alternatively, the catalyst is the enzyme, but formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject. This can be achieved by tagging the enzyme with specific carbohydrates or other liver cell specific structures for specific liver uptake, *i.e.* the enzyme (or analogue) is modified so as to facilitate active transport into e.g. liver cells.

10

Although the above embodiments are interesting, it is believed that the normal, practical embodiment of the invention will involve use of a catalyst which exerts substantially all its enzymatic activity extracellularly in the bloodstream, since it is believed that the metabolic products of the enzymatic conversion of the relevant heme precursor will permeate freely into the intracellular compartment where the remaining conversions of the heme biosynthetic pathway can take place. Alternatively, the metabolic product may be excreted from the subject via urine and/or faeces at least to some extent.

15

20 As mentioned above, it is preferred that the catalyst is produced recombinantly, *i.e.* by a method comprising

a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;

25 b) transforming a compatible host cell with the vector;

c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and

d) recovering the expression product from the culture

and optionally subjecting the expression product to post-translational processing, such

30 as *in vitro* protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.



For relatively small catalysts (e.g. those constituted mainly of the active site of the enzyme), the catalyst can alternatively be prepared by liquid-phase or solid-phase peptide synthesis.

5 A more detailed explanation of the recombinant production of the model enzyme

PBGD is given in the detailed section hereinafter, but as mentioned herein the same considerations apply for all other peptide catalysts of the invention. One of the main advantages of producing the catalyst by recombinant or synthetic means is, that if produced in a non-human cell, the catalyst is free from any other biological material of human origin, thus reducing problems with known or unknown pathogens such as viruses etc.

The dosage regimen will normally be comprised of at least one daily dose of the catalyst, (preferably by the intravenous route). Normally 2, 3, 4 or 5 daily dosages will be necessary, but if sustained release compositions are employed, less than 1 daily dosage are anticipated.

The daily dosage should be determined on a case by case basis by the skilled practitioner, but as a general rule, the daily dosage will be in the range between 0.01 – 1.0 mg/kg body weight per day of the catalyst. More often the dosage will be in the range of 0.05 – 0.5 mg/kg body weight per day, but it should never be forgotten that precise dosage depends on the dosage form and on the activity of the catalyst as well as on the degree of deficiency of the relevant enzyme and an individualized treatment, where the dose is adjusted to normalize patient serum and urine precursor levels.

25 The most correct way of determining the correct dosage is based on the patient specific precursor levels. The precursor being the product of the enzymatic reaction.

For PBGD, the daily dosage is about 0.08-0.2 mg per kg body weight per day, and most often 0.1 mg per kg body weight per day will be the dosage of choice. It is believed that comparable dosages will be applicable for the other full-length enzymes.

Finally, as will be appreciated from the above disclosure, the invention is based on the novel idea of providing substitution for the enzymes lacking in activity. To the best of

the knowledge of the inventors, therapeutic use of catalysts having such effects have never been suggested before, and therefore the invention also pertains to a catalyst as defined herein for use as a pharmaceutical. Furthermore, use of such catalysts for the preparation of pharmaceutical compositions for treatment of the above-discussed

5 diseases is also part of the invention.

Legends to figures:

Figure 1: Circular map of PBGD clone # 1.1 in pBluescript SK

10

Figure 2: Circular map of expressed plasmid pExp0

Figure 3: Circular map of expressed plasmid pPBGD 1.1

15

Figure 4: Circular map of expressed plasmid pKK223-3

Figure 5: Circular map of expressed plasmid pExp1

Figure 6: Circular map of expressed plasmid pPBGD 1.1 Tra

20

Figure 7: Circular map of expressed plasmid pExp1-M2

Figure 8: Circular map of expressed plasmid pExp1-M2-Puc-BB

25 Figure 9a-9x: PBGD clone # 1.1 in pBluescript SK - Sequence

## DETAILED DISCLOSURE OF THE INVENTION

30 In a first embodiment the invention relates to a method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalyst which is said enzyme or an enzymatically equivalent

part or analogue thereof. The disease may be selected from the porphyria group and  
~~the catalyst may be an enzyme selected from the group consisting of~~

porphobilinogen deaminase (PBGD)

ALA dehydratase,

5 Uroporphyrinogen decarboxylase,

Coproporphyrinogen oxidase,

Coproporphyrinogen oxidase,

Protoporphyrinogen oxidase,

Uroporphyrinogen III synthase,

10 Ferrochelatase, and

Uroporphyrinogen decarboxylase,

or an enzymatically equivalent part or analogue thereof.

In a preferred embodiment, the disease is AIP and the enzyme is PBGD or an

15 enzymatically equivalent part or analogue thereof. In a further embodiment, the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.

The catalyst may be administered by a route selected from the group consisting of the

20 intravenous route, the intraarterial route, the intracutaneous route, the subcutaneous route, the oral route, the buccal route, the intramuscular route, the anal route, the transdermic route, the intradermal route, and the intratechal route.

The catalyst is preferable formulated in an isotonic solution, such as 0.9% NaCl and

25 10-50 mM Sodium phosphate pH 7.0 +/- 0.5 up to pH 8.0 or Sodium phosphate, glycine, mannitol or the corresponding potassium salts. The catalyst may also be lyophilised, sterile filtered, and in a further embodiment formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof. In a still other embodiment the catalyst is incorporated into erythrocyte ghosts.

30

Also a sustained release formulation may be performed involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

A further method according to the invention is wherein the catalyst is lyophilized in a two-compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment. The two compartment cartridge may be combined with an injection device to administer the catalyst either by a needle or  
5 by a needle-less (high pressure) device.

It may also be very convenient to administer the catalyst in a formulation of a physiological buffer containing an enhancer for nasal administration.

10 Other formulations for the catalyst include an oral formulation containing lipid vesicles, such as those comprising phosphatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.

The formulation is preferable one which is able to enhance the half-life of the catalyst  
15 in the subject's bloodstream. This may be by use of a formulation wherein the catalyst has a polyethylene glycol coating.

The catalyst may also be complexed with a heavy metal.

20 In a further aspect the catalyst is an enzymatically equivalent part or analogue of the enzyme and exerts at least part of its enzymatic activity intracellularly upon administration to the subject. This may be when the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane.

25 Furthermore, the catalyst may be said enzyme formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

30 In addition the catalyst may be tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.

In a further aspect the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.

In a still further aspect, the enzymatic activity of the catalyst on its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.

A further embodiment of the invention relates to a method wherein the catalyst has been prepared by a method comprising

- 10 a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
- b) transforming a compatible host cell with the vector;
- c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
- 15 d) recovering the expression product from the culture and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino acid residues, and deglycosylation, so as to obtain the catalyst.
- 20 The catalyst may be prepared by liquid-phase or solid-phase peptide synthesis and it is preferable free from any other biological material of human origin.

As mentioned above the catalyst may be administered at least once a day, such as 2, 3, 4, and 5 times daily depending on the specific treatment regimen outlined for the 25 patient in that precursor levels for each patient are measured before and/or during treatment for evaluation of the specific dosage.

Accordingly the daily dosage may be in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day. And the 30 present invention also relates to the use of the catalyst for the preparation of a pharmaceutical composition.

It is estimated that a dosage will often be about 0.1 mg per kg body weight per day.

Accordingly, the invention also relates to a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof, for use as a medicament. Thus in a further embodiment, the invention relates to a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent

5 part or analogue thereof for the preparation of a pharmaceutical composition for the treatment or prophylaxis of diseases caused by deficiency of said enzyme.

Naturally, the catalyst may be a recombinant form of the enzyme. An example is a recombinant human PBCD based on any of Seq. ID NO 1 (clone PBGD 1.1) and Seq.

10 ID NO 12 (non-erythro PBGD 1.1.1).

In a preferred embodiment and as will be disclosed in detail below, the invention also relates to a method for treating a patient having a mutation in the PBGD gene causing an enzyme defect, the method comprising use of a human PBGD cDNA sequence of  
15 either non-erythropoietic form or erythropoietic form according to the tissue in which PBGD should be expressed, and transfecting the patient with the relevant cDNA. Preferably the enzyme deficiency is selected from enzyme deficiencies resulting in a disease selected from Acute Intermittent Porphyria (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP),  
20 Harderoporphyria (HDP), Variegata porphyria (VP), Congenital erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

In a preferred embodiment, the human PBGD cDNA sequence is selected from Seq. ID NO 1 (clone PBGD 1.1) and Seq. ID NO 12 (non-erythro PBGD 1.1.1).

25 The transfection may be by use of a vector selected from adenovirus, retrovirus and associated adenovirus. The PBGD gene transfer vector into human cells (erythropoietic and/or non-erythropoietic) preferable results in normal PBGD activity or in an activity wherein the patient is free of symptoms of disease.

30 A further method of gene therapy treatment of patients with Acute Intermittent Porphyria (AIP) according to the invention is by a correction of one of the specific point mutations identified causing AIP by use of chimeraplasty gene repair. This

involve specific designed oligonucleotides and a specific knowledge of both the mutation to be corrected and to the sequence on both sides on the mutation.

In a specific embodiment of chimeraplasty gene repair is by use of a delivery system

- 5 for transfection by use of non-viral vectors formulated in a vehicle preparation comprising one or more components selected from cationic phospholipids, phospholipids, phospholipids mixed with neutral lipids, lictosylated PEI, liposomes liposomes comprising mixtures of natural phopholipids and neutral lipids.

- 10 The mutation may be selected from the mutations shown in Table A.

The following description of preferred embodiments of the invention will focus on recombinant production of PBGD and formulations and uses thereof. It will be appreciated, however, that all disclosures relating to this polypeptide apply also for

- 15 the other enzymes mentioned above. Hence, production and use of PBGD only exemplifies the invention, but all other enzymes of the heme biosynthetic pathway can substitute PBGD in the embodiments described hereinafter.

#### Production of recombinant PBGD

20

As mentioned above, it is preferred to administer recombinant versions of the various enzymes of the heme biosynthetic pathway. In the following will be described recombinant production of one of these enzymes, namely PBGD.

- 25 The gene for the erythropoietic PBGD, which is located in the human genome in the chromosomal region 11q 24, is composed of 15 exons spanning 10 kb of DNA and is shown in Grandchamp B. et al. 1996. J. of Gastroenerology and Hepatology 11, 1046-1052.

- 30 The gene coding the erythropoietic PBGD enzyme (344 amino acids) (Raich N. et al 1986, Nucleic. Acid. Res, 14, 5955-5968), will be cloned from a human erythropoietic tissue by reverse transcriptase or amplification by PCR (polymerase chain reaction) of the PBGD coding region.

The gene will be inserted in a plasmid and transformed into a suitable host cell (a bacterium such as *E. coli* and *B. subtilis*, a fungus such as *Saccharomyces*, or a mammalian cell line, such as CHO cells). The expression of the PBGD gene will be regulated by a promoter which is compatible with the selected host cell.

5

For bacterial production: An endogenous ATG sequence is located at the NH<sub>2</sub>-terminal end of the rPBGD structural gene for initiation of translation and cytoplasmic expression. Alternatively insert in front of the PBGD coding region a bacterial signal sequence for example an *E. coli* periplasmic enzyme signal peptide or a signal peptide  
10 from a secreted enterotoxin or endotoxin in *E. coli*, to obtain secretion in *E. coli*.

The plasmid used for production of rPBGD in *E. coli* was constructed in the following way:

#### 15 Construction of the rPBGD production plasmid

##### **Introduction:**

The erythropoietic expressed form of porphobilinogen deaminase (PBGD) (Raich N, et al Molecular cloning and complete primary sequence of erythrocyte  
20 porphobilinogen deaminase. Nucleic Acids Research 1986 14(15): 5955-67)  
was cloned and sequence determined. Two forms of PBGD are known. The erythropoietic form is expressed specifically in erythroid progenitors and the constitutive form is expressed in all cells (Grandchamp B, et al Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. Eur J Biochem. 1987 Jan; 162(1):105-10.  
25 mp et. al. 1987.) The two are expressed from the same gene and are identical except for the addition of 17 amino acids at the amino terminus of the constitutive form through alternative exon usage. It was decided to clone and express the erythropoietic form. There are three sequences for PBGD in the Genebank, the two isoforms mentioned above and the genomic sequence (Yoo HW, et al Hydroxymethylbilane synthase: complete genomic  
30 sequence and amplifiable polymorphisms in the human gene. Genomics 1993 Jan; 15(1):21-9). These all have nucleotide differences translating to amino acid changes. Before choosing a specific sequence to be expressed for a human therapeutic it was therefore necessary to determine what is the wild type allele. To accomplish this, PBGD cDNA clones were isolated and sequenced from a number of sources to define the most



common amino acid usage. Oligonucleotide primers were designed to amplify the coding region from cDNAs by Polymerase Chain Reaction (PCR) (Saiki RK, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985, Dec 20; 230(4732):1350-4). These were used to

5 isolate cDNAs from 5 sources of mRNA which were then cloned into a plasmid vector.

Eight of these clones were sequenced and along with the published sequences define a wild type allele, which should be the most common amino acid sequence in the population. This wild type allele will be used for protein expression.

## 10 Strategy:

A nested PCR strategy was devised to clone PBGD. The first primer set, (see Table 1)

Ico379 and Ico382, are 20mers that bind to sequence outside of the coding region.

Ico379 is specific for the 5' untranslated region of the mRNA (cDNA) of the erythropoietic form of PBGD. The binding site is in an exon region not expressed in the

15 constitutive form of the enzyme. Ico382 binds to the 3' untranslated region of both forms of PBGD. Internal to these are a second set of oligonucleotide primers to be used for the second round of PCR, Ico375 and Ico376, designed to distal ends of the PBGD coding region. Ico375 has 22 bases of sequence homologous to the 5' end of the coding region of the erythropoietic form of PBGD with the ATG start codon

20 followed by an EcoRI endonuclease cleavage site for cloning of the PCR product and 4 bases of sequence to ensure efficient restriction. Ico376 has 33 bases homologous to the 3' end of the PBGD coding region with 3 bases changed internally to introduce a MunI/MfeI endonuclease cleavage site through silent mutations and ending with the TAA stop codon. This restriction site will be used to easily introduce sequence

25 encoding a His-Tag to the DNA with oligonucleotide adapters or to enable other 3' modifications. Following the stop codon is a second stop codon to ensure good termination of translation and a HindIII endonuclease cleavage site for cloning the PCR product followed by 4 bases to ensure efficient restriction. The EcoRI and HindIII endonuclease cleavage sites introduced onto the ends of the PBGD PCR product ligate

30 into the respective unique restriction sites in the high copy number pBluescriptII SK- (Stratagene) vector for sequencing and will then be used to move the PBGD DNA into an *E. coli* expression vector for production of recombinant human porphobilinogen deaminase, rh-PBGD.

**PCR:**

Six cDNAs were used as a PCR source, spleen, bone marrow, lymph node, lung, whole brain and adipose tissue each from a different pool of human donors (produced by Donald Rao using BRL Superscript II with 500 ng Clontech poly-A RNA in 20  $\mu$ l

- 5 reaction volumes per manufacturers instructions except adipose which was made from 5  $\mu$ g of Clontech total RNA from a single donor). List of equipment and supplies used (see lists below). One  $\mu$ l of each cDNA (approximately 25ng) was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3 $\mu$ M each of lco379 and lco382 in 50  $\mu$ l reaction volumes. Two cycle PCR was used, with
- 10 an initial heat denaturation step at 94°C for 1' 40" then 28 cycles of 96°C for 16" and 68°C for 2'. A final extension of 6' at 74°C ensured that extension products were filled out. One fifth of the reaction was run out on a 1.2% agarose gel with 2  $\mu$ l of 6X ficol loading dye in 0.5X TBE buffer (Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982 ). The
- 15 predicted band of 1.1 kb. was observed by ethidium bromide staining with all sources but lung tissue cDNA. These bands were excised and DNA was isolated with Microcon-30 with micropure inserts (Amicon/Millipore) per manufacturers instructions and buffer exchanged with dH2O. One tenth of the recovered DNA was amplified with Advantage cDNA polymerase mix (Clontech) with 0.2mM dNTP (PE/ABI) and 0.3 $\mu$ M
- 20 each of the internal nested oligonucleotides (lco375 and lco376) at 0.3 $\mu$ M in 50 $\mu$ l reactions. Two cycle PCR was used again with an initial heat denaturation step at 94°C for 1' 40" then 2 cycles of 96°C for 16" and 68°C for 2' then 13 cycles of 96°C for 16" and 72°C for 2' with a final extension of 6' at 74°C. Ten  $\mu$ l of the 50  $\mu$ l reactions were run on a 1.2% agarose gel with 2  $\mu$ l 6X loading dye. The resulting
- 25 bands were of the expected size, 1.05 kb. The remainder of the PCR reactions were passed through Chromaspin-400 columns (Clontech) per manufacturers instructions to remove reaction components and to exchange buffer with TE (10mM Tris-HCl pH8.0/ 1mM EDTA). The DNA containing eluates were washed with dH2O and concentrated with Microcon-100 spin-filters (Amicon/Millipore) per manufacturers instructions.

30

**Cloning:**

The purified PBGD DNA was digested for 6 hours with 40 Units each of EcoRI and HindIII in EcoRI "U" buffer (New England Biolabs (NEB)) in 50  $\mu$ l reactions at 37°C. Enzymes were heat killed for 20 minutes at 68°C and reactions were spun in Microcon

100 spin-filters to remove small DNA end pieces, washed with dH<sub>2</sub>O and concentrated. One fifth of the resulting DNA was ligated with approximately 50 ng EcoRI and HindIII digested and twice gel purified pBluescriptII SK- (Stratagene) and 200 units T4 DNA ligase (NEB cohesive end units) for 15 hours at 16°C. The ligase

- 5 was heat killed at 75°C for 10 minutes. The reactions were then buffer exchanged with dH<sub>2</sub>O and concentrated in Microcon-100 spin filters and volumes taken up to 5  $\mu$ l with dH<sub>2</sub>O. One  $\mu$ l each was electroporated into 25  $\mu$ l DH10B Electromax cells (Gibco/BRL) at 2.5Kv/2000ohms/25 $\mu$ F in 0.1cm cuvetts with a BioRad electroporator. One ml of SOC medium (Gibco/BRL) was added and the cells were outgrown at 37°C
- 10 for one hour at 250 rpm. Cells were plated out on LB plates (Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold Spring Harbor Laboratory. 1982) with 150  $\mu$ g/ml ampicillin. The efficiency of all five were approximately twice background (vector ligated without insert.) Colony PCR was used to analyze 18 transformants of each electroporation for the presence of PBGD. An internal PBGD
- 15 specific primer (lco381) was used with a pBluescript specific primer (lco385) to both confirm identity and proper orientation in the vector. The 25  $\mu$ l reactions were set up on ice to inactivate proteases with primer concentrations of 0.4 $\mu$ M, 0.125U Taq polymerase (Fisher), and 0.2mM dNTP(PE/ABI.) Two cycle PCR was used, with an initial heat denaturation step at 94°C for 1' 40" a further denaturing step at 96°C for
- 20 20 seconds, then 30 cycles of 96°C for 16" and 68°C for 1' with a final extension of 4' at 74°C. Five  $\mu$ l of 6X loading dye was added and 12.5  $\mu$ l each were run out on a 1.2% agarose gel. Results are as follows: 12/18 positive colonies for spleen; 10/18 for bone marrow; 8/18 for lymph node; 9/18 for brain and 10/18 for adipose tissue. Two positive colonies each for the first 3 and 1 each for the latter two were grown up in
- 25 25 ml. liquid LB culture with 150  $\mu$ g/ml ampicillin over night at 37°C with 250 rpm. Plasmid DNA was purified from the cultures with Qiagen's Tip-100 DNA purification kit per manufacturer's instructions. UV absorbance at 260nm was used to determine the plasmid yields which varied from between 131 and 169  $\mu$ g of highly purified DNA.

### 30 Sequencing:

Sequencing reactions of double stranded plasmid DNA with Big Dye terminator cycle sequencing were performed in a 9700 thermocycler (Perkin Elmer/Applied Biosystems.) Two vector primers (lco383 and lco385) and two PBGD specific internal primers (lco380 and lco381) were used for all 8 plasmids (see sequence). In addition a fifth

vector primer (lco285) was used for the brain and adipose derived clones. Reaction conditions were per manufacturers protocol as follows: 500 ng plasmid DNA and 4 pmol oligonucleotide primer with 8  $\mu$ l ready mix in 20  $\mu$ l volumes with 30 cycles of 96°C for 12" and 60°C for 4'. Extension products were purified by isopropanol precipitation. To each reaction 20  $\mu$ l of dH<sub>2</sub>O and 60  $\mu$ l Isopropanol were added. These were mixed by inversion and allowed to sit at room temperature for 15 minutes then spun for 40' at 3250 rpm in a Beckman GS-6KR centrifuge with the GH3 rotor and Microplate + carriers. Reactions were inverted then spun at 1680 rpm for 1' to remove liquid from the pelleted DNA. DNA sequence analysis was performed at the University of Washington Biochemistry Department sequencing Laboratory with an Applied Biosystems 377 sequencer.

#### Analysis:

The inserts of all 8 clones were confirmed to be PBGD by complete double strand sequence analysis (see sequences 1 - 8). Each has some change(s) from the published sequences. Some changes are unique and some are shared with other clones (see Table 2 and Table 3). For differences found only in one clone, it is difficult to distinguish between PCR or cloning artifacts and actual allelic variations without additional sampling. However, when the same base difference is found in more than one sequence it is unlikely to be from cloning errors. From the alignment of all 11 PBGD sequences a set of common bases emerged, the consensus or wild type allele sequence. Five of the eight clones (1.1, 1.3, 2.1, 3.3, and 5.3.) have the wild type amino acid sequence. Within this set with wild type amino acid sequence, there is only one difference at the nucleic acid level. At position 555, 4 of the 5 sequences have a dGTP while 1 along with the published erythropoietic and genomic PBGD have a dTTP. These appear to be two common alleles, which result in no amino acid difference. There are 2 base changes between clone number 1.1 and the published erythropoietic PBGD. An adenine to guanine change at base 513 (Leu 171) is a silent mutation, which is also present in 9 out of the 11 sequences, compared. The second difference is a cytosine to adenine substitution at base 995 (Thr 332.) This is not a silent change, with a threonine to asparagine non-conservative mutation. It appears however that the difference is an error in the published erythropoietic PBGD sequence since all 10 other sequences have an adenine at this position. In addition to these natural variations, there are three additional silent mutations introduced during the cloning at positions

1017, 1018 and 1020 to create a Mun-I site for future manipulations. The PBGD gene was ligated into pBluescript SK plasmid generating the pSK-PBGD 3988 bp plasmid, which was sequenced (see Fig. 1, Fig. 9a-9x and sequence 9).

## 5 Conclusion:

For any recombinant therapeutic protein it is important that the wild type allele be used to reduce the potential for immunogenicity. We feel confident through our survey of the literature and analysis of PBGD sequence from different individuals that clone number 1.1 represents the most prevalent "wild type" allele in the population with respect to amino acid sequence. Clone number 1.1 contains the consensus wild type amino acid sequence and differs from the published erythropoietic PBGD sequence by only one amino acid. Because this difference is found in all the other PBGD clones besides the erythropoietic PBGD sequence, it, rather than the published erythropoietic sequence, is deemed to be the prevalent wild type sequence. For this reason PBGD encoded by clone number 1.1 was chosen for production of recombinant human porphobilinogen deaminase (rh-PBGD.)

**Table 1 Oligonucleotide primers:**

5	<b>Ico375-pbgds</b> (32 mer) coding region 5' end w/ EcoRI site sense 5' CGT GGA ATT CAT GAG AGT GAT TCG CGT GGG TA 3'
	<b>Ico376-pbgda</b> (47 mer) coding region 3' end w/ HindIII site antisense 5' GGA GAA GCT TAT TAA TGG GCA TCG TTC AAT TGC CGT GCA ACA TCC AG 3'
10	<b>Ico378-csnonc</b> (20 mer) constitutive form non-coding sense 5' TCC AAG CGG AGC CAT GTC TG 3'
	<b>Ico379-esnonc</b> (20 mer) erythropoietic form non-coding sense 5' TCG CCT CCC TCT AGT CTC TG 3'
15	<b>Ico380-sinter</b> (21 mer) internal coding sense 5' CAG CAG GAG TTC AGT GCC ATC 3'
20	<b>Ico381-ainter</b> (21 mer) internal coding antisense 5' GAT GGC ACT GAA CTC CTG CTG 3'
	<b>Ico382-anonc</b> (20 mer) non-coding sense 5' CAG CAA CCC AGG CAT CTG TG 3'
25	<b>Ico383-pSKT7</b> (22 mer) pBluescript T7 promoter 5' GTA ATA CGA CTC ACT ATA GGG C 3'
	<b>Ico384-pSKpjrev</b> (22 mer) pBluescript reverse1 5' CTA AAG GGA ACA AAA GCT GGA G 3'
30	<b>Ico385-pSKrev</b> (21 mer) pBluescript reverse2 5' CAG CTA TGA CCA TGA TTA CGC 3'

**Equipment list:**

Item	Manufacturer	Serial Number
Pipetman P-1000	Gilson	N55287E
Pipetman P-200	Gilson	N52324E
Pipetman P-20	Gilson	N53465M
Pipetman P-10	Gilson	P626586
5415C centrifuge	Eppendorf	5415B68381
GS-6KR centrifuge	Beckman	NGD97J18
Avanti J-25 I centrifuge	Beckman	JJY97J14
DU 640B Spectrophotometer	Beckman	4323015
Genie II vortex	VWR	2-241186
GeneAmp PCR system 2400	Perkin Elmer (PE) / Applied Biosystems (ABI)	803N6021903
GeneAmp PCR system 2400	PE / ABI	803S7100104
GeneAmp PCR system 9700	PE / ABI	805S7121566
1545 incubator	VWR	0902597
heat block 1	VWR	0795
heat block 1	VWR	0511
Gene Pulser II Apparatus	BioRad	340BR2745
Pulse Controller Plus	BioRad	339BR1377
Power Pac 1000	BioRad	286BR00770
Sub Cell	BioRad	16S/8860
Wide-Mini Sub Cell	BioRad	02S/7951
Foto/Prep transilluminator	Fotodyne	PTG1-0997-2831
Elutrap Electro-separator	Schleicher + Schuell	Order No. 57880
Innova 4000 incubator	New Brunswick Scientific	890165366
Power Mac G3 computer	Macintosh	XA8061A3BBW
Trinitron Multiscan 200GS monitor	Sony	8057052
DNA analysis Software: Geneworks	Intelligenetics	Version 2.5.1

**Supplies List:**

Item	Supplier	Cat No.	Lot No.
Human Spleen Poly A + RNA	Clontech	6542-1	7120266
Human Bone Marrow Poly A + RNA	Clontech	6573-1	56714
Human Lung Poly A + RNA	Clontech	6524-1	7050104
Human Lymph Node Poly A + RNA	Clontech	6594-1	6120292
Human Brain Poly A + RNA	Clontech	6516-1	63101
Human Adipose Total RNA	Clontech	D6005-01	7907005
Superscript II reverse transcriptase	Gibco/BRL	18064-014	JM6418
100 mM dNTP set	Pharmacia	27-2035-01	6072035011
pBluescriptII SK- phagemid	Stratagene	212206	0270702
Advantage cDNA polymerase mix	Clontech	8417-1	8060354
GeneAmp dNTP	PE/ABI	N-808-0007	H0172.4,H0553
Xba-I endonuclease	New England Biolabs (NEB)	145S	30
Pvu-II endonuclease	NEB	151L	14
EcoR-I endonuclease	NEB	101L	25
Hind-III endonuclease	NEB	104S	49
Tris six-Pack "C"	Sigma	T-PAC-C	77H9049
0.5 M EDTA pH 8.0	Sigma	E-7889	16H8924
Chromaspin TE 400	Clontech	K1323-1	7090795
Chromaspin 400 DepC dH2O	Clontech	K1333-1	7040086
Quiaquick gel extraction kit	Qiagen	28704	BY97017/0397/119
Microcon-30	Amicon	42410	L8JM4330B
Microcon-100	Amicon	42413	L8DM3296A



Micropure 0.22µm	Amicon	42544	CCB017
Seakem GTG agarose	FMC	50074	709397
100 bp DNA Ladder	NEB	323-1	3
123 bp DNA Ladder	Gibco/BRL	15613-029	JK9706
T4 DNA Ligase	NEB	202S	64
Ampicillin	Sigma	A-9518	76H0434
LB media	Gibco/BRL	12795-084	12E1072B
Bacto Agar	Difco	0140-07-4	106728JA
DH10B electromax	Gibco/BRL	18290-015	KHN430
SOC media	Gibco/BRL	15544-042	1010351
Taq polymerase	Fisher	FB-6000-15	H0436
TaqStart antibody	Clontech	5400-1	6070479
Qiafilter Midi DNA isolation kit	Qiagen	12243	PO No. 514
Isopropanol	Sigma	I-9516	47H3724
Big Dye terminator cycle sequencing kit	PE/ABI	4303152	9803008

**Table 2 Variation of PBGD clones from published erythroid sequence:**

PBGD clone	Differences from Erythroid mRNA			Genebank No.	Reference/Source
	silent	non-silent	total diffs		
Erythroid	0	0	0	X04217	Raich,N. et. Al. Nucleic Acids Res. 14 (15), 5955-5968 (1986)
Constitutive	1	2	3	X04808	Grandchamp,B. et. Al. Eur. J. Biochem. 162 (1), 105-110 (1987)
Genomic	1	2	3	M95623	Yoo,H.W. et. Al. Genomics 15 (1), 21-29 (1993)
1.1	1	1	2	-	Spleen (Clontech mRNA Lot No. 7120266)
1.3	2	1	3	-	Spleen (Clontech mRNA)
2.1	2	1	3	-	Bone Marrow (Clontech mRNA)
2.2	2	2	4	-	Bone Marrow (Clontech mRNA)
3.1	2	4	6	-	Lymph Node (Clontech mRNA)
3.3	3	1	4	-	Lymph Node (Clontech mRNA)
5.3	2	1	3	-	Total Brain (Clontech mRNA)
6.1	3	2	5	-	Adipose Tissue (Clontech mRNA)

**5 Table 2:**

Summary of the number of differences in amino acid sequence of our sequenced PBGD clones and clones from Genebank entrys for the constitutive and genomic PBGD with published Erythropoietic PBGD sequence. Shown in different columns are the total number of silent mutations with a DNA base change not causing a corresponding amino acid change, the number of non-silent mutations with a DNA change causing an amino acid difference and the sum of the two types of mutations. Not included in this figure are the three silent mutations introduced into the clones to create an internal Mun-I endonuclease cleavage site. Note that clone number 1.1 which will be used for production of recombinant human porphobilinogen deaminase (rh-PBGD) has only one of each type of difference with the least number of total differences.

Table 3 Summary of mutations found in PBGD clones:

aa	aa No.	bp No.	mutation	aa change	cons.	gen.	1.1	1.3	2.1	2.2	3.1	3.3	5.3	6.1	No. /10
Asp	19	56	A -> G	Asp -> Gly							X				1
Phe	108	322	T deletion	frame shift						X					1
Lys	140	419	A -> G	Lys -> Arg							X				1
Leu	160	478	C -> A	Leu -> Met		X									1
Ala	168	503	C -> T	Ala -> Val											1
Leu	171	513	A -> G	silent		X	X	X	X	X					1
Val	185	555	T -> G	silent	X			X	X			X	X	X	9
Glu	193	577	G -> A	Glu -> Lys	X		X	X				X	X	X	7
Gly	243	729	C -> T	silent								X			1
Ala	280	840	T -> C	silent											1
Ala	286	856	G -> A	Ala -> Thr										X	1
Lys	328	984	A -> G	silent										X	1
Thr	332	995	C -> A	Thr -> Asn	X	X	X	X	X	X	X				1
Gln	339	1017	G -> A	silent			X	X	X	X	X	X	X	X	10
Gln	339	1018	C -> T	silent			X	X	X	X	X	X	X	X	8
Leu	340	1020	T -> G	silent			X	X	X	X	X	X	X	X	8
Leu	340	1020	T deletion	frame shift									X		7
															1

Table 3:

Summary of the genetic differences of our sequenced PBGD clones and Genebank entries for the constitutive and genomic PBGD with published erythropoietic PBGD sequence from the allele sequence alignment. Listed in different columns are the amino acid, base number from the ATG start codon, the actual genetic difference with corresponding amino acid change if any and a listing of the clones with differences shown with an X. The final column tallies the number of clones with that mutation. The final four mutations are introduced with lco376 during PCR amplification to create a Mun-I endonuclease cleavage site. Note that clone number 1.1 which will be used for production of rh-PBGD only has differences which are represented by a number of clones.

## Expression plasmids

The expression plasmid pExp0 (see Fig. 2) was constructed by excising the PBGD coding sequence (nt1-1043) from pPBGD1.1 (see Fig. 3) with *EcoR* I and *Hind* III and inserting it into the vector pKK223-3 (see Fig. 4) (Pharmacia Cat # 27-4935, Lot # 8054935011) cut with the same enzymes, thus operatively linking it to the inducible tac promoter. Another plasmid, pExp1 (see Fig. 5) was also constructed with modifications to the 5' untranslated region and the initial part of the coding sequence both aimed at improving translation efficiencies (translation enhanced). The changes are indicated below and include, insertion of a second ribosome binding site, an AT rich sequence preceding the ATG and three silent base substitutions shown in as underlined.

AATTCTAACA TAAGTTAAGG AGGAAAAAAA A ATG AGA GTT ATT CGT GTC GGT AC

Met Arg Val Ile Arg Val Gly

Plasmid pExp1 (Fig. 5) was made in a two step process. Oligonucleotides ICO386 (5' AAT TCT AAC ATA AGT TAA GGA GGA AAA AAA AAT GAG AGT TAT TCG TGT CGG TAC 3') and ICO387 (5' CGA CAC GAA TAA CTC TCA TTT TTT TTT CCT CCT TAA CTT ATG TTA G 3') were designed to provide upon annealing a 5' *EcoR* I adhesive end and a 3' *Kpn* I sticky end. Oligonucleotides ICO386 and ICO387 were annealed and ligated with the *Kpn* I-*Hind* III PBGD fragment from pPBGD1.1 into *EcoR* I-*Hind* III linearized pBluescript II SK- (Stratagene Cat # 212206) to yield plasmid pPBGD1.1Tra (Fig. 6). In the second step the *EcoR* I-*Hind* III fragment from pPBGD1.1Tra was ligated into pKK223-3 cut with the same enzymes resulting in plasmid pExp1 (Fig. 5).

## Host strains and growth conditions

For the studies done so far two *E. coli* K12 strains were used. Both these strains carry the *lacI<sup>q</sup>* repressor and are, DH12S (Life Technologies cat # 1832-017) and JM105 (Pharmacia Cat # 27-1550-01). The media used was mainly LB Broth (Difco Laboratories Cat # 0446-07-5) containing 100 µg/ml ampicillin. The cultures were grown at 37° C and induced with 4mM IPTG for 3 hours.

The choice of the final strain will partly depend on the induction strategy used (see below). The use of *lacI<sup>q</sup>* will force the use of strains that do not carry the *lacI<sup>q</sup>* repressor. Further, strains deficient in proteases such as La, Clp and the *rpoH* locus may prove to be beneficial if thermal induction is to be used.

The hemC gene is deleted in the final production strain. A plasmid, which will be used as a tool for this purpose has been constructed.

#### 5 Restoration of tetracycline resistance gene

Plasmid pExp1-M2 (Fig. 7) has been constructed from pExp1 with a functional tetracycline resistance gene. This was designed with a view to be able to introduce other regulatory elements or genes as required by the project as also being able to retain the ability to replace the "translation enhanced" PBGD sequence by the native sequence or a polylinker  
10 for the blank plasmid.

The following strategy was used. Plasmid pExp1 was cut with *Sal* I and *Bam* H I and the 5348 base-pair fragment containing part of the tetracycline coding sequence and the bulk of the plasmid was isolated. Into this was ligated the *Sal* I-*Hind* III fragment from pBR322  
15 (New England BioLabs Cat # 303-3S, Lot # 50) containing rest of the coding sequence and an adapter formed by annealing oligonucleotides ICO424 (5' GATCACTCAT GTTTGACAGC TTATCATCGA TT 3') and ICO425 (5. AGCTAATCGA TGATAAGCTG TCAAACATGA GT 3'). The adapter contains part of the tetracycline promoter and provides  
20 *Hind* III and *Bam* H I overhangs for ligation but destroys the *Hind* III and *Bam* H I restriction sites.

#### Induction:

25 The tac promoter is a strong promoter when induced with IPTG and other groups have shown that it or the other almost identical trc promoter can be induced very well at 42°C when a thermosensitive lac repressor is used. With pExp1-M2-Puc-BB construct a high level of expression which can translate to gram quantities of product in the fermenter. Based on study data and data from the literature the thermal induction is efficient.

30

*Strategy 1:* The plasmid pExp1 (see Fig. 5) has a basal level of expression which is about one-fifth that of a fully induced culture in a *lacI<sup>q</sup>* strain in LB broth. Stable and with acceptable expression levels, it can be used without induction in defined fermentation media. In the plasmid pExp1-M2 (see Fig. 7) the rom gene has been deleted on the  
35 plasmid and the pBR223 replicon exchanged by the pUC-based replicon. These plasmids

have elevated copy numbers with increasing temperature (greater than 30°C) and serve as a pseudo-induction system.

- Strategy 2: In plasmid pExp1-M2 a thermo-labile lacI repressor has been introduced either at the *Sca* I site or the *Bsa* I site. The repressor was derived from plasmid pTYB1 (New England BioLabs Cat # 6701, Lot # 3) by PCR, wherein Gly<sup>187</sup> was changed to Ser. If the thermal induction works but a high basal level of expression leads to instability, the native PBGD sequence as in pExp0 (without the enhancements) is substituted. In plasmid pExp0 the uninduced level of expression is below the level of detection in the assay system used.
- 10 Other variations would be to use lacI<sup>q</sup>ts on the plasmid or in the genome.

#### Detailed Description of the Construction of the production plasmid for rPBGD

- The production plasmid pExp1-M2-Puc-BB (see Fig. 8) for rPBGD was constructed in a multi-step process. The individual steps used and all the intermediate plasmids are outlined below.

- The expression plasmid pExp1 was first constructed wherein the PBGD coding sequence is operatively linked to the tac promoter of the vector pKK223-3 (Pharmacia Cat # 27-4935, Lot # 8054935011) by means of a short adapter designed to include a ribosome binding site, an AT-rich region preceding the ATG and the first six codons of the PBGD human erythropoietic enzyme with three silent base changes. The sequence of the adapter is shown below with the base changes introduced in the wobble positions boldfaced.

AATTCTAACA TAAGTTAAGG AGGAAAAAAA A ATG AGA<sup>~</sup>GT<sup>T</sup> ATT CG<sup>T</sup> GT<sup>C</sup> GGT AC

Met Arg Val Ile Arg Val Gly

- Oligonucleotides ICO386 (5' AAT TCT AAC ATA AGT TAA GGA GGA AAA AAA AAT GAG AGT TAT TCG TGT CGG TAC 3') and ICO387 (5' CGA CAC GAA TAA CTC TCA TTT TTT TTT CCT CCT TAA CTT ATG TTA G 3') were designed to provide upon annealing a 5' *Eco*R I adhesive end and a 3' *Kpn* I sticky end. Oligonucleotides ICO386 and ICO387 were annealed and ligated with the *Kpn* I-*Hind* III PBGD fragment from pPBGD1.1 into *Eco*R I-*Hind* III linearized pBluescript II SK- (Stratagene Cat # 212206) to yield plasmid pPBGD1.1Tra. In the second step the *Eco*R I-*Hind* III fragment from pPBGD1.1Tra was ligated into pKK223-3 cut with the same enzymes resulting in plasmid pExp1.

The tetracycline resistance gene was next restored using the following strategy.

Plasmid pExp1 was cut with *Sal* I and *Bam* H I and the 5348 base-pair fragment containing part of the tetracycline coding sequence and the bulk of the plasmid was isolated. Into this  
5 was ligated the *Sal* I-*Hind* III fragment from pBR322 (New England BioLabs Cat # 303-3S, Lot # 50) containing rest of the coding sequence and an adapter formed by annealing oligonucleotides ICO424 (5' GATCACTCAT GTTTGACAGC TTATCATCGA TT 3') and ICO425 (5. AGCTAATCGA TGATAAGCTG TCAAACATGA GT 3'). The adapter contains part of the tetracycline promoter and provides *Hind* III and *Bam* H I overhangs for ligation  
10 but destroys the *Hind* III and *Bam* H I restriction sites. The resulting plasmid was called pExp1-M2.

Plasmid pExp1-M2 was digested with *Pvu* I and *Afl* III and the larger of the two fragments corresponding to a size of 4745 base-pairs was isolated. This was ligated to the 1257  
15 base-pairs long *Pvu* I-*Afl* III fragment derived from pUC19 containing the origin of replication and part of the ampicillin resistance gene to obtain plasmid pExp1-M2-Puc. This was passaged through JM110 and cut with *Bsa* A1 and *Bsa* B1 to excise the *rom* gene contained between the two sites and blunt-ended together to yield the final expression plasmid pExp1-M2-Puc-BB. The pExp1-M2-Puc-BB plasmid has been fully sequenced (see  
20 sequence 11).

### Expression of rPBGD in *E. coli*

The *E. coli* K12 host strain JM105 genotype endA thi rpsL sbcB15 hsdR4  $\Delta$ (lac-proAB) [F'*tra*D36 proAB lacI<sup>q</sup>  $\Delta$ (lacZ)M15 ] containing the expression plasmid pExp1-M2-Puc-BB  
25 was grown in LB broth containing 100  $\mu$ g/ml ampicillin at to mid-log phase at 30°C from a 1 to 40 dilution of an overnight inoculum. The culture was then split into three and growth was continued for another 4 hours at 30°C, 37°C and 42°C respectively. Cells were spun down from 1 ml samples and frozen at -20°C. The thawed cell pellets were resuspended in 200-300  $\mu$ l of B-PER reagent PIERCE Cat. # 78243, incubated at room temperature for 10  
30 minutes, spun at 16,000 for 10 minutes and PBGD activity was determined in the supernatants. Total protein was estimated by the Bradford method using the BioRad reagent Cat # 500-0006 and bovine serum albumin as standard. The specific activities in the crude lysates obtained at the three growth temperatures are tabulated below. The results clearly show an increase of PBGD units/mg with increasing temperature in the  
35 range from 30°C to 40°C.

Temperature	PBGD Units/mg
30°C	363
37°C	573
5 42°C	1080

## OTHER PRODUCTION SYSTEMS FOR rPBGD

10 For yeast production, the PBGD coding sequence can be inserted into a plasmid vector, for example YEP type, containing 2 u circular DNA (Ori) origin for high expression in yeast. YEP plasmids contain TRP 1 and URA 3 as markers for selective maintenance in trp1-, ura 3-yeast strains.

15 Alternatively, the PBGD gene can be inserted in bovine papilloma virus vectors BPV for high expression in a murine cell line C-127 (Stephens P.E. et. al. Biochem J. 248, 1-11, 1987) or vectors compatible with expression in CHO cells or COS cells.

An expression of PBGD can be made intracellularly.

20

A secretory signal in Saccharomyces, for example alpha-mating factor presequence, can be added in front of the rPBGD structural gene for efficient secretion in yeast.

25 Similarly, a sequence encoding a mammalian signal peptide can be added for secretion of rPBGD into the culture medium upon expression in for example CHO cells or COS cells.

A bacterial promoter for example the tryptophane (trp) promoter or the lac promoter or alternatively an alkaline phosphatase promoter, should be inserted before the PBGD  
30 coding region for efficient transcription in prokaryotes for example E. coli.

A yeast promoter for example 3-phosphoglycerate kinase (PGK) or chelatin or alpha-mating factor should be inserted before the PBGD coding region for efficient



transcription in yeast for example *Saccharomyces cerevesiae* or *Saccharomyces pombe*.

A mammalian promoter for example Metallothionin-1 (MT-1) or Aspartate

- 5 transcarbamylase or Dihydrofolate reductase (DHFR) should be inserted before the PBGD coding region for efficient transcription in mammalian cell lines for example CHO cells or COS cells.

The yeast plasmid (Y-G&F-PBGD) containing a yeast promoter, signal and/or ATG

- 10 codon (methionine) in front of the PBGD coding region and a yeast vector containing selectable markers such URA 3 or TRP 1 will be transformed into the yeast host cell such as *Saccharomyces cerevesiae* or *Saccharomyces pombe* for production of rPBGD.

- 15 The mammalian plasmid (M-G&F-PBGD) containing a mammalian promoter for example Metallothionine-1 or Dihydrofolate reductase and a mammalian signal sequence or an ATG codon in front of the PBGD coding region and vector pAT or pSV2 respectively. Plasmid (M-G&F PBGD) will be transfected into a mammalian cell line for example CHO cells, for production of rPBGD.

- 20 The *E. coli* cell containing plasmid (pExp1 or pExp1-M2 Puc-BB), will be fermented to stationary phase between 24-48 hours, in a medium containing casein hydrolysate, or yeast extract, glucose, vitamins and salts. pH oxygen will be monitored by electrodes during fermentation. Temperature will be kept at  $37 \pm 2$  C during the fermentation.

- 25 The yeast cell containing the plasmid (Y-G&F-PBGD), will be fermented to late log phase between 20-40 hours in a medium containing yeast extract, glucose, salts and vitamins. pH and temperature will be monitored by specific electrodes during fermentation. Temperature will be kept at  $30 \pm 2$  C during fermentation.

- 30 The mammalian cell line containing the plasmid (M-G&F-PBGD) will be fermented in a medium containing, foetal calf serum (or serum free), vitamins, glucose, antibiotics, growth factors. pH and temperature will be monitored continuously during fermentation by specific electrodes.

### Fermentation and Purification

rPBGD will be recovered from *E. coli* after fermentation by an extraction procedure involving for example ribipress, sonication, osmotic shock or total solubilization by

5 detergent for example Tween 80, Triton X-100 or Brij. rPBGD will be recovered from fermentation medium after production in yeast or from a total cellular extract using detergents such as Triton X-100, Tween 80 or Brij. rPBGD will be recovered from mammalian culture medium or from a total cellular extract by ion-exchange chromatography or affinity chromatography.

10 rPBGD will be purified from *E. coli* extract or from yeast medium or total cellular extract or from mammalian culture medium or total mammalian cellular extract by binding to an ion-exchange column for example DEAE-Sepharose or MonoQ-Sepharose and eluted with for example NaCl and Sodium phosphate buffer pH 7-8 or the  
15 corresponding potassium salts.

Alternatively, rPBGD will be recovered from extracts by binding to an affinity chromatography column for example an anti-PBGD affinity column. rPBGD will be eluted by lowering the pH to 4-2, or a thiol specific affinity column. rPBGD has been  
20 "tagged" with thiol residues when a thiol affinity column step is used. Thiols will be removed by a specific enzymatic cleavage step to generate authentic rPBGD.

The ion-exchange or affinity purified rPBGD will be further purified by hydrophobic interaction chromatography on for example, TSK Phenyl 5 PW column or Octyl-  
25 Sepharose or Phenyl-Sepharose columns.

Binding of rPBGD will be done at high ionic strength for example in 10-50 mM Tris-HCl pH 7-8, 1M NaCl or 10-15 mM Sodium phosphate pH 7-8, 0.5 M  $\text{MgSO}_4$  and eluted by lowering the ionic strength for example with 10-50 mM Tris-HCl pH 7-8 or  
30 10-50 mM Sodium phosphate pH 7-8.

Three hydrophobic interaction steps will be applied consecutively.

rPBGD will be further purified with preparative RP-HPLC for example C12 or C18 matrixes. The rPBGD will be eluted from the column by a gradient of 10-50 mM Sodium phosphate and 1-10% acetonitrile buffer.

5 Formulation of rPBGD will be done by passing the enzyme over a G-100 Sephadex column and eluting it in an isotonic solution for example 0.9%NaCl and 10-50mM Sodium phosphate pH7.0 +/- 0.5 or Sodium phosphate, glycin, mannitol or the corresponding potassium salts.

10 The formulation solution of rPBGD will be sterile filtered and filled aseptically in glass vials and lyophilised.

Alternatively, the sterile filtered rPBGD solution will be formulated in for example, lipid vesicles constituting phosphatidylcholine or phosphatidylethanolamine or combinations

15 of these or incorporated into erythrocyte ghosts.

Reconstitution of lyophilised rPBGD will be done in water for injection.

Alternatively, rPBGD will be formulated in a sustained release formulation involving a  
20 biodegradable microspheres, for example in polylactic acid, polyglycolic acid or mixtures of these.

Alternatively, rPBGD will be lyophilized in a two-compartment cartridge, where rPBGD will be in the front compartment and water for reconstitution in the rear compartment.

25 This two compartment cartridge will be combined with an injection device to administer either rPBGD by a needle or needle less (by high pressure) device.

Alternatively, rPBGD will be formulated in a physiological buffer containing an enhancer for nasal administration.

30

Alternatively, rPBGD will be formulated in an oral formulation containing for example, lipid vesicles (phosphatidylcholine, phosphatidylethanolamine, sphingomyeline) or dextrane microspheres.

Although recombinant production of PBGD is preferred for the treatment of AIP, it can alternatively be produced from human red blood cells.

The production and manufacturing of recombinant PBGD will be done by the following 5 steps.

#### Recombinant PBGD production process; an outline

##### A: Fermentation

- 10 1. Master cell bank
2. Working cell bank
3. Production of seed culture
4. Fermentation in large fermenter (250 L >)

##### 15 B. Purification

1. Cell concentration by filtration/centrifugation
2. Cell disruption
3. Ultrafiltration
4. Chromatography ion exchange, DEAE-Sepharose, MonoQ-Sepharose
- 20 5. Hydrophobic interaction chromatography (Octyl/phenyl-Sepharose, TSK Phenyl, 5PW, Phenyl -Sepharose
6. Chromatography ion exchange (MonoQ)
7. Formulation by Gel filtration Sephadex G-100

##### 25 C. Manufacturing

1. Sterile filtration
2. Aseptic filling
3. Lyophilization

## TREATMENT OF OTHER PORPHYRIAS

In analogy with the new treatment of AIP patients with (recombinant) PBGD, hepatic Porphyrins such as ALA deficiency Porphyria (ADP), Porphyria cutanea tarda (PCT),

- 5 Hereditary Coproporphyria (HCP) and Variegata Porphyria (VP) can benefit from substitution therapy by rALA dehydratase, rUroporphyrinogen decarboxylase, rCoproporphyrinogen oxidase and rProtoporphyrinogen oxidase, respectively.

- 10 Patients having Erythropoietic Porphyrins such as Congenital erythropoietic Porphyria (CEP) or Erythropoietic protoporphyria (EPP) will benefit from substitution therapy with rUroporphyrinogen III syntetase and rFerrochelatase, respectively.

Hepatoerythropoietic Porphyrins e.g. Hepatoerythropoietic Porphyrins (HEP) can be treated with rUroporphyrinogen decarboxylase.

15

All porphyrias can be treated by the administration of the enzymatic activity lacking or being reduced (normally 50%) in any of the eight steps in the heme biosynthetic pathway as described above.

- 20 The substitution of the enzymatic activity can be achieved by adding the corresponding recombinant enzyme or other molecules that will provide the missing enzymatic activity.

## GENE THERAPY AS AN ALTERNATIVE TREATMENT POSSIBILITY FOR PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA (AIP)

25

The human enzyme Porphobilinogen deaminase PBGD is coded for by a single gene located on chromosome 11q 24.

- 30 Mutations in this gene causes the disease Acute Intermittent Porphyria (AIP). The disease has been shown to be inherited in an autosomal dominant way.

- Today over 100 mutations in the PBGD gene has been identified (Grandchamp B. J. Ganstroenterology and Hepathology, 11, 1046-1052, 1996, Table A) and the number is expected to increase when modern diagnostic systems based on screening programs will  
35 be applied more routinely in hospitals. A number of these mutations are shown in Table A

Table A Reported mutations in the PBGD gene

	Position	Mutation	Consequences	Reference
Exon 1	3	ATG→ATA	Translation impairment	18
	33	GCG→GCT	DS	17
<i>Intron 1</i>	33 + 1	gtg→atg	DS	16
Exon 3	76	CGC→TGC	R26C	25
	77	CGC→CAC	R26H	26
Exon 4	91	GCT→CACT	A31T	24
	97	Del A	Frameshift	25
	100	CAG→AAG	Q34K	27
	100	CAG→TAG	Q34X	25
	125	TTG→TAG	L42X	19
Exon 5	163	GCT→TCT	A55S	24
	174	Del C	Frameshift	24
	182	Ins G	Frameshift	24
<i>Intron 5</i>	210 + 1	gta→ata	DS (Del exon 5)	24
Exon 6	218-219	Del AG	Frameshift	24
Exon 7	277	GTT→TTT	V93F	24
	293	AAG→AGG	K98R	25
	331	GGA→AGA	G111R	28
<i>Intron 7</i>	345-1	cag→caa	AS (Del exon 8)	29
Exon 8	346	CGG→TGG	R116W	20
	347	CGG→CAG	R116Q	30
Exon 9	445	CGA→TGA	R149X	25
	446	CGA→CAA	R149Q	31
	446	CGA→CTA	R149L	24
	463	CAG→TAG	Q155X	32
	470	Ins A	Frameshift	29

Table A cont.

	Position	Mutation	Consequences	References
<i>Intron 9</i>	499-1	cag→caa	AS (Del exon 10)	21
<i>Exon 10</i>	499	CGG→TGG	R167W	33
	500	CGG→CAG	R167Q	27,34
	518	CGG→CAG	R173Q	34
	530	CTG→CGG	L177R	27
	593	TGG→TAG	W198X	19
	604	Del G	Frameshift	35
	610	CAG→TAG	Q204X	30
	612	CAG→CAT	DS (Del 9 bp exon 10)	31
<i>Exon 11</i>	625	GAG→AAG	E209K	28
<i>Intron 11</i>	652-3	cag→gag	AS (Del exon 12)	33
<i>Exon 12</i>	667	GAA→AAA	E223K	24
	673	CGA→GGA	R225G	25
	673	CGA→TGA	R225X	25
	713	CTG→CGG	L238R	25
	715-716	Del CA	Frameshift	19
	730-731	Del CT	Frameshift	36
	734	CTT→CGT	L245R	31
	739	TGC→CGC	C247R	36
	740	TGC→TTC	C247F	18
	742	Ins 8 bp	Frameshift	24
	748	GAA→AAA	E250K	24
	754	GCC→ACC	A252T	36
	755	GCC→GTC	A252V	36
	766	CAC→AAC	H256N	27
	771	CTG→CTA	DS (Del exon 12)	39
	771	CTG→CTC	DS (Del exon 12)	37
<i>Intron 12</i>	771+1	gta→ata	DS (Del exon 12)	19

Table A cont.

	Position	Mutation	Consequences	References
Exon 13	806	ACA→ATA	T269I	30
	820	GGG→AGG	G274R	30
Exon 14	838	GGA→AGA	G280R	25
	848	TGG→TAG	W283X	30
	886	CAG→TAG	Q296X	25
	900	Del T	Frameshift	31
Intron 14	912 + 1	gta→ata	DS (Del exon 14)	28
Exon 15	1062	Ins C	Frameshift	38
	1073	Del A	Frameshift	25

- 5 In one aspect, the present invention relates to a therapeutic method for AIP patients based on gene therapy.

The gene therapy treatment may involve the following steps.

- 10 1. Identification mutations in the PBGD gene causing AIP in humans  
 2. Selection of human PBGD cDNA sequence for gene therapy  
 3. Construction of PBGD gene therapy vectors.  
 4. Production of PBGD gene transfer vector  
 5. Delivery system of PBGD gene transfer vector

15 1. Identification of mutations in the PBGD gene causing AIP in humans

Patients having a point mutation in Exon 10 at position 593 TGG>TAG have a change in the amino acid sequence of the PBGD enzyme from W198X (stop codon). This mutation is  
 20 carried by approximately 50 % of all AIP patients in Sweden (Lee JS. et al. Proc. Natl. Acad. Sci. USA, 88, 10912-10915, and 1991). AIP patients with other mutations than W198X, which might also benefit from gene therapy, are given in Table A.

2. Selection of human PBGD cDNA sequence for gene therapy



There are two isoenzyme forms of human PBGD e.g. erythropoietic and the non-erythropoietic form, which are formed by an alternative splicing mechanism.

The non-erythropoietic form has a 17 amino acid extension on the N-terminal end of the erythropoietic PBGD form.

5

#### **Non-erythropoietic PBGD form (nPBGD):**

Met-Ser-Gly-Asn-Gly-Asn-Ala-Ala-Ala-Thr-Ala-Glu-Glu-Asn-Ser-Pro-Lys-Met-Arg-Val....

ATG-TCT-GGT-AAC-GGC-ATT-GCG-GCT-GCA-ACG-GCG-GAA-GAA-AAC-AGC-CCA-AAG-ATG-

10 AGA-GTG..

#### **Erythropoietic PBGD form (ePBGD):**

**Met-Arg-Val-**

ATG-AGA-GTG...

15

The nucleotide and amino acid sequence for human PBGD that will be used for gene therapy differs from that published by Raich N. et al. Nucl. Acid. Res. 14, 5955-5968, 1986 in that the amino acid residue in position 332 is an Asn residue rather than Thr. In order to make the "wild type enzyme" and avoiding formation of antibodies the PBGD sequence has to contain an Asn residue in position 332. The cDNA sequence that will be used for the erythropoietic PBGD form is shown above.

20

Patient with a defect erythropoietic PBGD enzyme will be transfected with the erythropoietic PBGD cDNA sequence and patients with a defect in the non-erythropoietic form will be transfected with the non-erythropoietic cDNA sequence.

25

### 3. Construction of PBGD gene therapy vectors

30

#### **Adenoviral vector system**

The vector is based on adenovirus type 5 (Ad5), containing three essential genetic loci E.g. E1, E2, E4, encoding important regulatory proteins and one locus E3 which is non-essential for virus growth. Deletion of E1A and E1B region renders the virus replication

deficient in vivo. Efficient complementation of the E1 function (recombinant viral stocks) can be obtained in an E1 expressing cell line such as human 293-cell line.

The human PBGD cDNA will be inserted in an adenovirus vector system.

The PBGD transgenes will be driven by the endogenous PBGD promoter or a cytomegalovirus promoter (CMV).

### Retroviral vectors

10 Retroviral vectors are well suited for gene delivery for several reasons:

1. simplicity
2. capacity to integrate up to 8kbp DNA inserts
3. their safety, non pathogenic to humans
- 15 4. easy to improve and manipulate
5. defined integration sites of genes
6. long term regulated expression

One major disadvantage with the retroviral vectors though, is that they can only transduce  
20 dividing cells.

Most common retroviridae considered for gene therapy, are the lentiviridae and the mammalian C-type viridae. Other type retroviruses have also been considered. One such example, is a Moloney-murine leukemia retrovirus (Mo-MLV), which has been successfully  
25 used to transduce mouse and human fibroblasts with the uroporphyrinogen III synthetase (UROIIIIS). (Moreau-Gaudry et al. Human Gene Therapy 6, 13-20, 1995).

The expression of the UROIIIIS gene was driven by long terminal repeat (LTR). The UROIIIIS cDNA was also successfully transduced by the retrovirus vectors into human  
30 peripheral blood progenitor cells.

The erythropoietic PBGD cDNA sequence can be inserted in a retrovirus vector LXSN (Miller et al BioTechniques 7, 980-990, 1989) and pMFG ( Dranoff et al. Proc. Natl. Acad.Sci. USA. 90,3539-3543, 1993). This will lead to the following constructs e.g. LePSN  
35 and pMFG-ePBGD, respectively.

**LePSN:**

> 1032 bp <

5 LTR-----/ cDNA ePBGD / SV40 / Neo /-----LTR

**pMFG-ePBGD:**

> 1032 bp <

10 LTR-----/ cDNA ePBGD/-----LTR

For transduction of non-erythropoietic tissues the non-erythropoietic cDNA (See sequence 12) will be inserted in the LSXN vector and the pMFG vector resulting in the LSnPN and pMFG-nPBGD vectors, respectively.

**LnPSN:**

> 1083 bp <

LTR-----/ cDNA nPBGD / SV40 / Neo /-----LTR

**pMFG-nPBGD:**

> 1083 bp <

LTR-----/ cDNA nPBGD/-----LTR

The LePSN and LnPSN vectors can be converted to the corresponding virus by transfer into an appropriate host cell line e.g.  $\Psi$  CRE as described by (Danos et al. Proc. Natl. Acad. Sci. USA. 85, 6460-6464, 1988). Filtered supernatants from ectopic virus producing cells were added to amphotropic cells  $\Psi$  CRIP, in the presence of Polybrene. Clones can be isolated and tested for virus. Clones that show titers over 1.000.000 cfu/ml will be saved (resistant to G418).

The LnPSN vector will be cotransfected with the pMCI-Neo plasmid (Pharmacia, Sweden) into the packaging cell line  $\Psi$  CRIP. Clones that shows integration of provirus and high expression levels of message will be selected.

Filtrate from supernatants from virus producing cells (erythropoietic PBGD form) can be mixed with Polybrene and incubated with peripheral blood progenitor cells (bone marrow transplant) from an AIP patient for several hours. The transduced progenitor cells can then be transplanted back into an AIP patient.

The success of the treatment will be measured as the increase in the PBGD activity in erythrocytes and reduced excretion of ALA and PBG in the urine. Clinically a success of the treatment can be evaluated as a reduction of frequency of spontaneous acute attacks or drug-induced attacks. This will be a more convenient way of administering the recombinant PBGD enzyme than regular injections. The efficacy of the therapy can be evaluated by measuring the PBGD activity in blood and reduced excretion of PBG and ALA in the urine. Clinically, a successful treatment should result in less number of acute attacks or preferably no more attacks.

#### 15 **Associated Adenovirus system (AAV)**

AAV is a non-pathogenic human virus (Parvovirus) carried by more than 80% of all people. The advantage with AAV as compared to retroviral systems is that AAV can transduce both dividing and non-dividing cells. The virus genome, which is small, contains two Inverted Terminal Repeats (ITR) and a REP and CAP functions. The REP and CAP functions can be deleted and exogenous cDNA inserted.

Construction of an AAV vector containing the erythropoietic PBGD cDNA can be made. This AAV/PBGD vector will be suitable to transduce AIP patient's bone muscle cells, as a "muscle factory" for PBGD enzyme production. The PBGD cDNA will be engineered in such a way that a signal sequence for secretion will be added on the 5'-end of the cDNA. This will allow the erythropoietic PBGD enzyme to become secreted from the muscle cells into the blood stream. By this system patients will receive a constant delivery of active PBGD enzyme into the bloodstream, which will metabolize PBG thereby avoiding acute attacks.

#### - Non-erythropoietic

Alternatively, liver cells can be transduced with AAV containing the non-erythropoietic PBGD cDNA. The construct will be engineered in such a way that the translated PBGD enzyme will remain intracellular e.g. contain a Met residue at the N-terminal end of the PBGD enzyme without a signal sequence for secretion in mammalian cells. The PBGD transgene will be transcribed and translated into new PBGD enzymes that will remain intracellularly. Levels of new PBGD enzymes made in the liver will be normalized the

PBGD activity to 100%. AIP patients have usually reduced PBGD activity (50-80%) in the liver depending on the mutation and individual variations.

This treatment would alleviate the clinical symptom e.g. acute attacks with abdominal pain and reduce excretion of PBG and ALA in the urine. The AAV containing the non-erythropoietic PBGD form can also be used to correct the genetic defect in other cell types such as neuronal tissue, pancreas spleen e.g. non-erythropoietic tissue, by a similar mechanism.

#### 10 - Erythropoietic

The erythropoietic PBGD cDNA can be inserted in an AAV vector and used to transduce erythropoietic cells and stem cells in AIP patients, having a mutation affecting the erythropoietic form of PBGD.

#### 15 4. Production of PBGD gene transfer vector

Adenovirus have approximately 36 kbp double stranded DNA, containing three essential early gene loci (E1, E2, and E4) encoding important regulatory proteins. Loci E3 codes for a gene product that block immune response to virus infected cells *in vivo*. The PBGD gene transfer adenovirus vector can be produced by deleting the E1 and E3 loci. The PBGD gene cassette is inserted in that position instead. The virus will be replication defective when the E1 locus has been deleted. Efficient E1 complementation and thus high yield of recombinant virus vector (PBGD) can be obtained in an E1 expressing cell line, such as the human 293 cell line. (Graham, F. et al. 1977, Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. 36, 59-72).

#### 5. Delivery systems of PBGD gene transfer vectors.

Delivery of viral vectors are based on injection into the patient of a virus particle that will transduce human cells *in vivo*.

#### **Correction of point mutations causing AIP by Chimeraplasty Gene Repair**

The basic technique involves the synthesis of chimeric (RNA-DNA) oligonucleotides. The oligonucleotide will repair point mutations on the chromosome by binding to the site of

mutation and create a mismatch. The endogenous "mismatch repair system" which is present in all living cells, will correct the mutation.

The Chimeric oligonucleotides has the following general properties:

- 5 a. 68 mer (65-70 is acceptable size)
- b. 25 base DNA stretches at the 5'-end homologous to the normal sequence of the gene
- c. the 25 base DNA is designed in such a way the 12 bp on each side of the mutation is complementary to "wild-type DNA" where the mutation to be altered is located at position 13
- 10 d. the 25 mere contains 4 T bases at the one end to loop back the oligo to the other DNA strand with a 25 base sequence homologous to the other strand of the chromosomal DNA.
- e. the second strand is chimeric in that it contains 10 homologous bases of 2'O methyl RNA followed by 5 bases of DNA (containing a central mismatch e.g. correction of the human point mutation by mismatch repair) followed by another stretch of 10 bases of homologous 2'O methyl RNA. This stretch of DNA/RNA is followed with 5 bases of GC clamp and 4 T bases to form the second loop and finally a 5 base CG clamps complementary to the other one.

## 20 EXAMPLE 1

Correction of the PBGD mutation at position 593 TGG>TAG resulting in W198X

### Normal Chromosomal Sequence:

25 5'-AG CGC ATG GGC TGG CAC AAC CGG GT-3'  
Gln Arg Met Gly Trp His Asn Arg Val

### AIP Chromosomal Sequence:

5'- AG CGC ATG GGC TAG CAC AAC CGG GT-3'  
30 Stop

The sequence of the chimeric oligonucleotide ( Heme593W/X) is:

# DNA

5

G

RNA

↓

↓

↓

RNA

Chromosome ..... 5'- AG CGC ATG GGC TAG CAC AAC CGG GT-3'.....

gggccTC GCG TAC CCG ACC GTG TTG GCC CA

T

T

10

(

)

loop

T

Heme593W/X

T

loop

T

T

(

)

Tcccg

T

15

5'-AG CGC ATG GGC TGG CAC AAC CGG GT

Chromosome ..... 3'-TC GCG TAC CCG ATC GTG TTG GCC CA -5'.....

↑

C

20 The same principle of chimeric oligonucleotide can be constructed to correct any of the mutations causing AIP depicted in Table A.

Chimeric oligonucleotides can be used to correct any other point mutation causing any of the 8 known Porphyrias in a similarly as described above.

25

## Delivery of PBGD gene transfer of non viral vectors to humans

The chimeric oligonucleotide can be formulated in a vehicles preparation containing  
30 anionic or cationic phospholipids or phospholipids mixed with neutral lipids or lictosylated PEI.

Alternatively, the non-viral vectors can be formulated in liposomes containing mixtures of natural phospholipids and neutral lipids.

35

Specific protein sequences can be incorporated into liposomal membranes, that recognizes cellular receptors for specific targeting of non-viral vectors to a specific cell type such as liver, neuronal tissue or erythropoietic tissues, can be incorporated. Alternatively specific antibodies recognizing specific cellular surface antigens can be used for targeting. Thirdly, carbohydrates on the liposomal membrane can be used for liver uptake of chimeric oligonucleotides.

The formulated chimeric oligonucleotide (HemeBiotech 595 W/X) will be administered by sc. or IV. injections to AIP patients.

The efficacy of the treatment can be evaluated as above.

## GENE THERAPY AS AN ALTERNATIVE TREATMENT OF OTHER PORPHYRIC DISEASES

The gene therapy strategies outlined herein can also be used for other Porphyric diseases. The general principle is to increase the cellular or systemic content of a particular defective enzyme causing the disease. The following Porphyric diseases can be encompassed by this strategy:

1. ALA deficiency porphyria (ADP)
2. Porphyria cutanea tarda (PCT)
3. Hereditary coproporphyria (HCP)
4. Harderoporphyria (HDP)
5. Variegata porphyria (VP)
6. Congenital erythropoietic porphyria (CEP)
7. Erythropoietic protoporphyria (EPP)
8. Hepatoerythropoietic porphyria (HEP)



# References:

Andersson, Christer, Thesis, 1997, ISBN 91/7191/280/0, pp. 22-23

5 Danos et al. Proc. Natl. Acad. Sci. USA. 85, 6460-6464, 1988

Dranoff et al. Proc. Natl. Acad. Sci. USA. 90,3539-3543, 1993

Grandchamp B, et al Tissue-specific expression of porphobilinogen deaminase. Two  
10 isoenzymes from a single gene. Eur J Biochem. 1987 Jan;162(1):105-10.

Grandschamp B. et al. 1996. J. of Gastroenerology and Hepatology 11, 1046-1052

Herrick A.L. et al 1989, Lancet 1, 1295-1297

15

Jeans J. et al. American J. of Medical Genetics 1996,65, 269-273

Lithner F. et al. 1984, Acta.Med.Scand. 215,271-274

20 Maniatis T., E.F. Fritsch, J. Sambrook. Molecular Cloning (A laboratory Manual) Cold  
Spring Harbor Laboratory. 1982.

Miller et al BioTechniques 7, 980-990, 1989

25 Moreau-Gaudry et al. Human Gene Therapy 6, 13-20,1995).

Mustajoki et al. 1989, Sem. Hematol. 26, 1-9).

Raich N, et al Molecular cloning and complete primary sequence of erythrocyte  
30 porphobilinogen deaminase. Nucleic Acids Research 1986 14(15): 5955-67

Raich N. et al 1986, Nucleic. Acid. Res, 14, 5955-5968

Saiki RK, et al Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985 Dec 20;230(4732):1350-4.

5 Sassa S. 1996, Blood Review, 10, 53-58

Stephens P.E. et. al. Biochem J. 248, 1-11, 1987

Strand et al. Proc. Natl. Acad. Sci. 1970, 67, 1315-1320

10

Tishler P.V: et al. 1985, Am.J.Psychiatry 142,1430-1436

Waldenström J. Acta.Med. Scand. 1937 Suppl.82

15 Welland F.H. et al. 1964, Metabolism,13, 232

Wetterberg L. 1967, Svenska bokförlaget Nordstedt,Stockholm

Wetterberg L. 1976, In Doss M. Nowrocki P. eds. Porphyrrias in Human Disease.

20 Reports of the discussion. Matgurg an der Lahn, 191-202

Yeung L. et al. 1983, Q J. Med 52, 92-98

Yoo HW, et. Al. Hydroxymethylbilane synthase: complete genomic sequence and  
25 amplifiable polymorphisms in the human gene. Genomics 1993 Jan; 15(1):21-9.

## CLAIMS

1. A method for treatment or prophylaxis of disease caused by deficiency, in a subject, of an enzyme belonging to the heme biosynthetic pathway, the method comprising administering, to the subject, an effective amount of a catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof.
2. A method according to claim 1, wherein the disease is selected from the group consisting of
- 10 acute intermittent porphyria (AIP),  
ALA deficiency porphyria (ADP),  
Porphyria cutanea tarda (PCT),  
Hereditary coproporphyria (HCP),  
Harderoporphyria (HDP),
- 15 Variegata porphyria (VP),  
Congenital erythropoietic porphyria (CEP),  
Erythropoietic protoporphyria (EPP), and  
Hepatoerythropoietic porphyria (HEP).
- 20 3. A method according to claim 1 or 2, wherein the catalyst is an enzyme selected from the group consisting of  
porphobilinogen deaminase (PBGD)  
ALA dehydratase,  
Uroporphyrinogen decarboxylase,
- 25 Coproporphyrinogen oxidase,  
Coproporphyrinogen oxidise,  
Protoporphyrinogen oxidase,  
Uroporphyrinogen III synthase,  
Ferrochelatase, and
- 30 Uroporphyrinogen decarboxylase,  
or an enzymatically equivalent part or analogue thereof.
4. A method according to any of the preceding claims, wherein the disease is AIP and the enzyme is PBGD or an enzymatically equivalent part or analogue thereof.

5. A method according to any of the preceding claims, wherein the catalyst is a recombinant form of the enzyme belonging to the heme biosynthetic pathway or of the enzymatically equivalent part or analogue thereof.

5 6. A method according to any of the preceding claims, wherein the catalyst is administered by a route selected from the group consisting of the intravenous route, the intraarterial route, the intracutaneous route, the subcutaneous route, the oral route, the buccal route, the intramuscular route, the anal route, the transdermic route, the intradermal route, and the intratechal route.

10

7. A method according to any of claims 1-6, wherein the catalyst is formulated in an isotonic solution, such as 0.9% NaCl and 10-50 mM Sodium phosphate pH 6.50 to 8 or Sodium phosphate, glycine, mannitol or the corresponding potassium salts.

15 8. A method according to any of claims 1-7, wherein the catalyst is lyophilised.

9. A method according to any of claims 1-8, wherein the catalyst is sterile filtered.

10. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is  
20 formulated as lipid vesicles comprising phosphatidylcholine or phosphatidylethanolamine or combinations thereof.

11. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is incorporated into erythrocyte ghosts.

25

12. A method according to any of claims 1-6, 8 or 9, wherein the catalyst is formulated as a sustained release formulation involving biodegradable microspheres, such as microspheres comprising polylactic acid, polyglycolic acid or mixtures of these.

30 13. A method according to any of claims 1-9, wherein the catalyst is lyophilized in a two-compartment cartridge, where the catalyst will be in the front compartment and water for reconstitution in the rear compartment.

14. A method according to claim 13, wherein the two compartment cartridge is combined with an injection device to administer the catalyst either by a needle or by a needle-less (high pressure) device.

5 15. A method according to any of claims 1-9, wherein the catalyst is formulated in a physiological buffer containing an enhancer for nasal administration.

16. A method according to any of the preceding claims, wherein the catalyst is formulated as an oral formulation containing lipid vesicles, such as those comprising  
10 phosphatidylcholine, phosphatidylethanolamine, or sphingomyeline, or dextrane microspheres.

17. A method according to any of the preceding claims, wherein the catalyst is formulated so as to enhance the half-life thereof in the subject's bloodstream.

15

18. A method according to claim 17, wherein the catalyst has a polyethylene glycol coating.

20

19. A method according to claim 17, wherein the catalyst is complexed with a heavy metal.

25

20. A method according to any of the preceding claims, wherein the catalyst is an enzymatically equivalent part or analogue of the enzyme and exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

21. A method according to claim 20, wherein the catalyst is a small artificial enzyme or an organic catalyst which can polymerize porphobilinogen to hydroxymethylbilane

22. A method according to any of claims 1-9, wherein the catalyst is said enzyme  
30 formulated in such a manner that it exerts at least part of its enzymatic activity intracellularly upon administration to the subject.

23. A method according to claim 22, wherein the catalyst is tagged with specific carbohydrates or other liver cell specific structures for specific liver uptake.

24. A method according to claims 1-19, wherein the catalyst exerts substantially all its enzymatic activity extracellularly in the bloodstream.

25. A method according to claim 24, wherein the enzymatic activity of the catalyst on  
5 its relevant heme precursor results in a metabolic product which 1) either moves into the intracellular compartment and is converted further via the remaining steps of the heme biosynthetic pathway or 2) is excreted from the subject via urine and/or faeces.

26. A method according to any of the preceding claims, wherein the catalyst has been  
10 prepared by a method comprising

- a) introducing, into a suitable vector, a nucleic acid fragment which includes a nucleic acid sequence encoding the catalyst;
- b) transforming a compatible host cell with the vector;
- 15 c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; and
- d) recovering the expression product from the culture  
and optionally subjecting the expression product to post-translational processing, such as in vitro protein refolding, enzymatic removal of fusion partners, alkylation of amino  
20 acid residues, and deglycosylation, so as to obtain the catalyst.

27. A method according to any of claims 1-25, wherein the catalyst has been prepared by liquid-phase or solid-phase peptide synthesis.

25 28. A method according to any of the preceding claims, wherein the catalyst is free from any other biological material of human origin.

29. A method according to any of the preceding claims, wherein the catalyst is administered at least once a day, such as 2, 3, 4, and 5 times daily.

30

30. A method according to any of the preceding claims wherein the daily dosage is in the range of 0.01 – 1.0 mg/kg body weight per day, such as in the range of 0.05 – 0.5 mg/kg body weight per day.

31. A method according to any of the preceding claims, wherein the daily dosage is about 0.1 mg per kg body weight per day.

32. A catalyst which is an enzyme of the heme biosynthetic pathway or an

5 enzymatically equivalent part or analogue thereof, for use as a medicament.

33. Use of a catalyst which is an enzyme of the heme biosynthetic pathway or an enzymatically equivalent part or analogue thereof for the preparation of a pharmaceutical composition for the treatment or prophylaxis of diseases caused by  
10 deficiency of said enzyme.

34. The use according to claim 33, wherein the treatment or prophylaxis is performed according to any of claims 1-31.

15 35. A method according to any of claims 1-31 wherein the catalyst is a recombinant form of the enzyme.

36. A method according to any of claims 1-31 wherein the catalyst is recombinant human PBGD based on any of Seq. ID NO 1 (clone PBGD 1.1) and Seq. ID NO 12  
20 (non-erythro PBGD 1.1.1).

36. A catalyst according to claim 32, which is recombinant human PBGD based on any of Seq. ID NO 1 (clone PBGD 1.1) and Seq. ID NO 12 (non-erythro PBGD 1.1.1).

25 37. A method for treating a patient having a mutation in the PBGD gene causing an enzyme defect, comprising the use of a human PBGD cDNA sequence of either non-erythropoietic form or erythropoietic form according to the tissue in which PBGD should be expressed, and transfection of the patient with the relevant cDNA.

30 38. The method according to claim 37 wherein the enzyme deficiency is selected from enzyme deficiencies resulting in a disease selected from Acute Intermittent Porphyria, (AIP), ALA deficiency porphyria (ADP), Porphyria cutanea tarda (PCT), Hereditary coproporphyria (HCP), Harderoporphyria (HDP), Variegata porphyria (VP), Congenital

erythropoietic porphyria (CEP), Erythropoietic protoporphyria (EPP), and Hepatoerythropoietic porphyria (HEP).

39. The method according to claim 39 wherein the disease is Acute Intermittent

5 Porphyria, (AIP).

40. The method according to any of claims 37-39 wherein the human PBGD cDNA sequence is selected from Seq. ID NO 1 (clone PBGD 1.1) and Seq. ID NO 12 (non-erythro PBGD 1.1.1)

10

41. The method according to any of claims 37-40 wherein the transfection is by use of a vector selected from adenovirus, retrovirus and associated adenovirus.

42. The method according to any of claims 37-41 wherein the PBGD gene transfer  
15 vector into human cells (erythropoietic and/or non-erythropoietic) results in normal PBGD activity.

43. A method of gene therapy treatment of patients with Acute Intermittent Porphyria (AIP) by a correction of one of the specific point mutations identified causing AIP by  
20 use of chimeraplasty gene repair.

44. The method according to any of claims 43 wherein the delivery system for transfection is by use of non-viral vectors formulated in a vehicle preparation comprising one or more components selected from cationic phospholipids, phospholipids,  
25 phospholipids mixed with neutral lipids, liposylated PEI, liposomes liposomes comprising mixtures of natural phospholipids and neutral lipids.

45. A method according to claim 43 or 44 wherein the mutation is selected from Table A.



# ABSTRACT OF THE DISCLOSURE

A method for treatment or prophylaxis of disease

caused by deficiency, in a subject, of an enzyme belonging to

the heme biosynthetic pathway, the method comprising

administering, to the subject, an effective amount of a catalyst

which is said enzyme or an enzymatically equivalent part or

analogue thereof. The disease is selected from the group

consisting of, acute intermittent porphyria (AIP), ALA

deficiency porphyria (ADP), Porphyria cutanea tarda (PCT).

Hereditary coproporphyria (HCP), Harderoporphyria (HDP),

Variegata porphyria (VP), Congenital erythropoietic porphyria

(CEP), Erythropoietic protoporphyria (EPP), and

Hepatoerythropoietic porphyria (HEP). The catalyst is an enzyme

selected from the group consisting of porphobilinogen deaminase

(PBGD) ALA dehydratase, Uroporphyrinogen decarboxylase,

Coproporphyrinogen oxidase, Coproporphyrinogen oxidase,

Protoporphyrinogen oxidase, Uroporphyrinogen III synthase,

Ferrochelatase and Uroporphyrinogen decarboxylase, or an

enzymatically equivalent part or analogue thereof. In addition

the invention relates to the use of PBGD and to a method of gene

therapy.

1/31

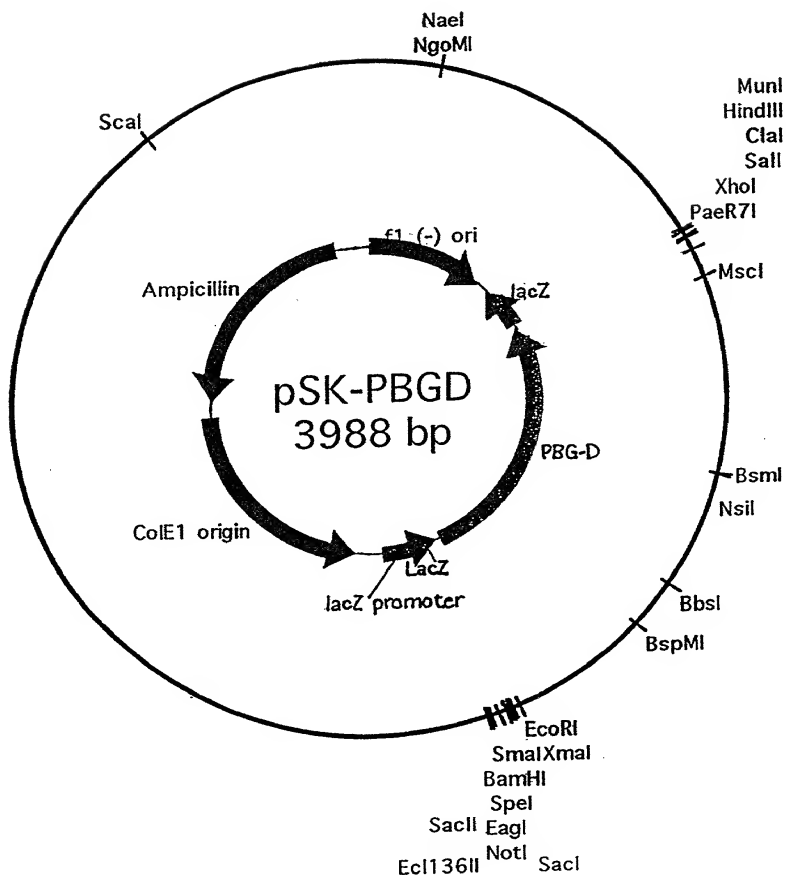


Fig. 1

2/31

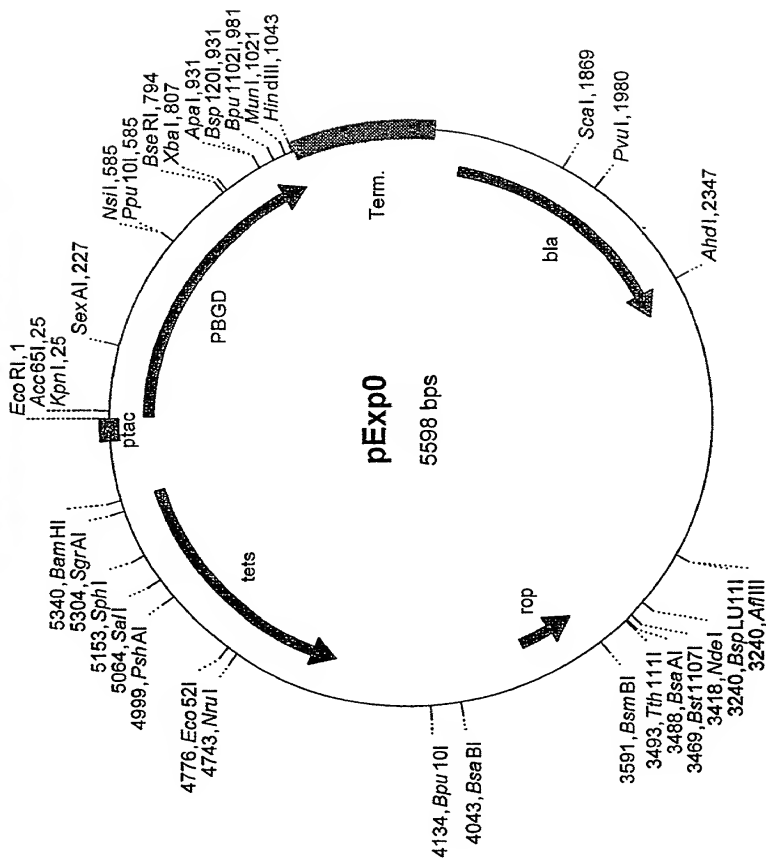


Fig. 2

09/601138

HA3

3/31

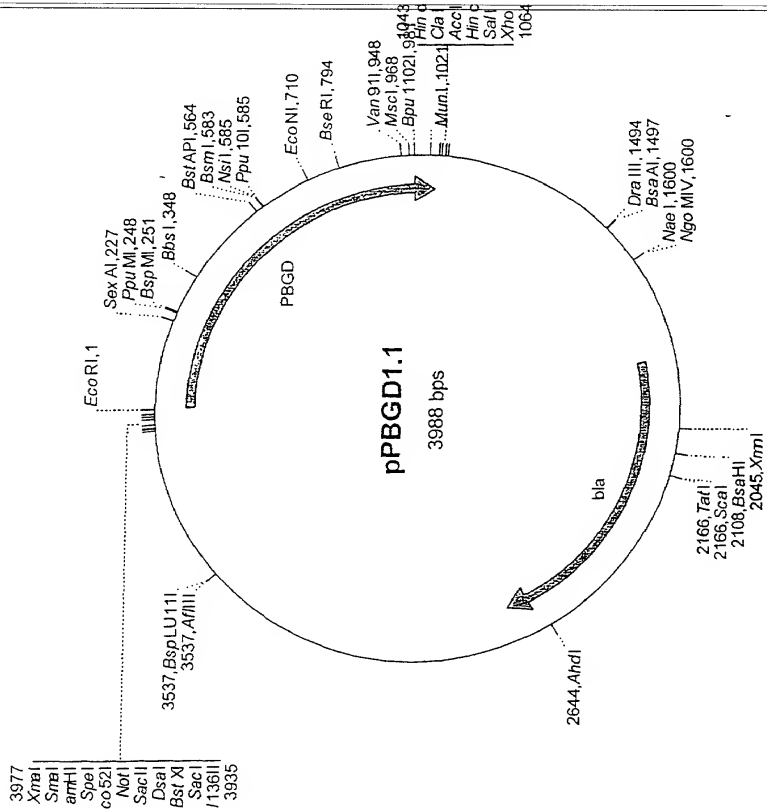


Fig. 3

4/31

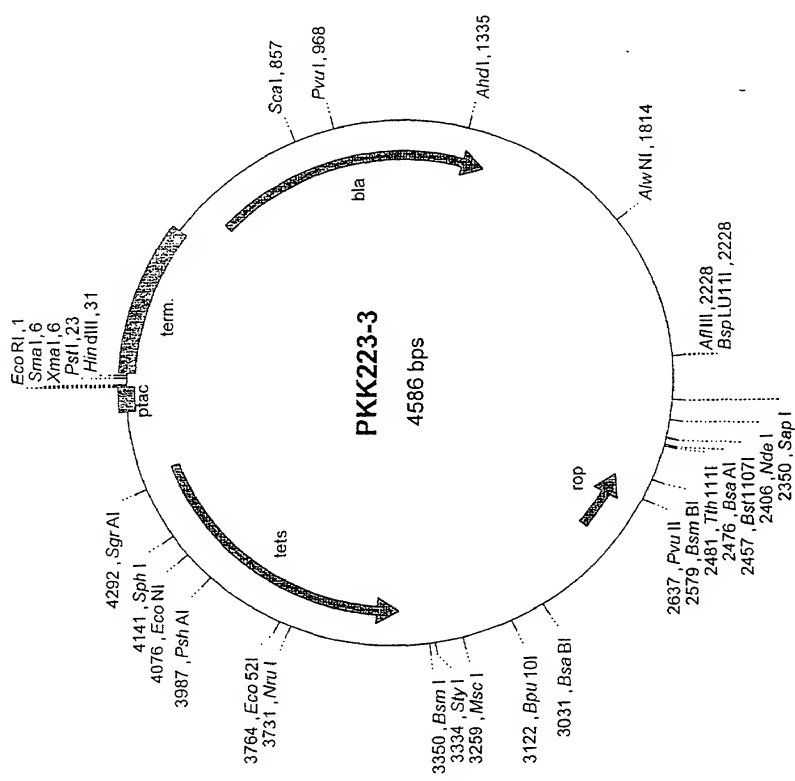


Fig. 4

5/31

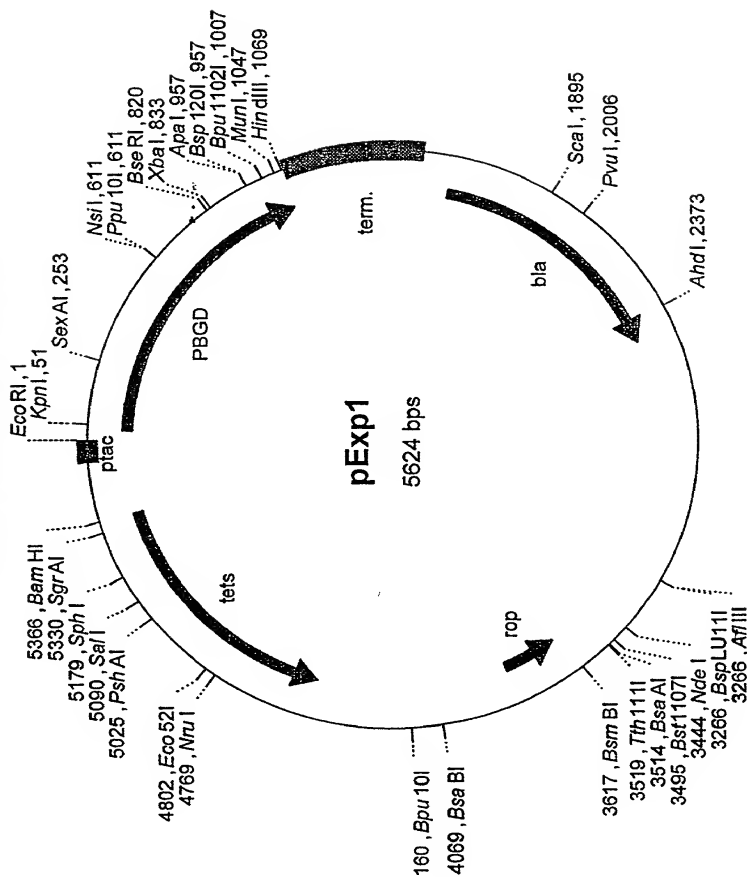
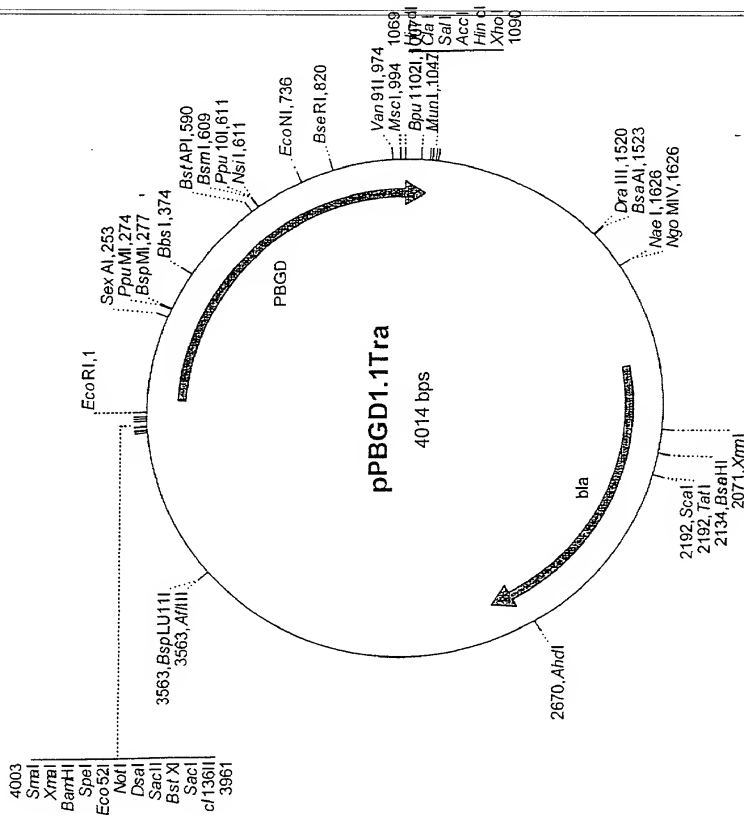


Fig. 5

6/31



**Fig. 6**

7/31

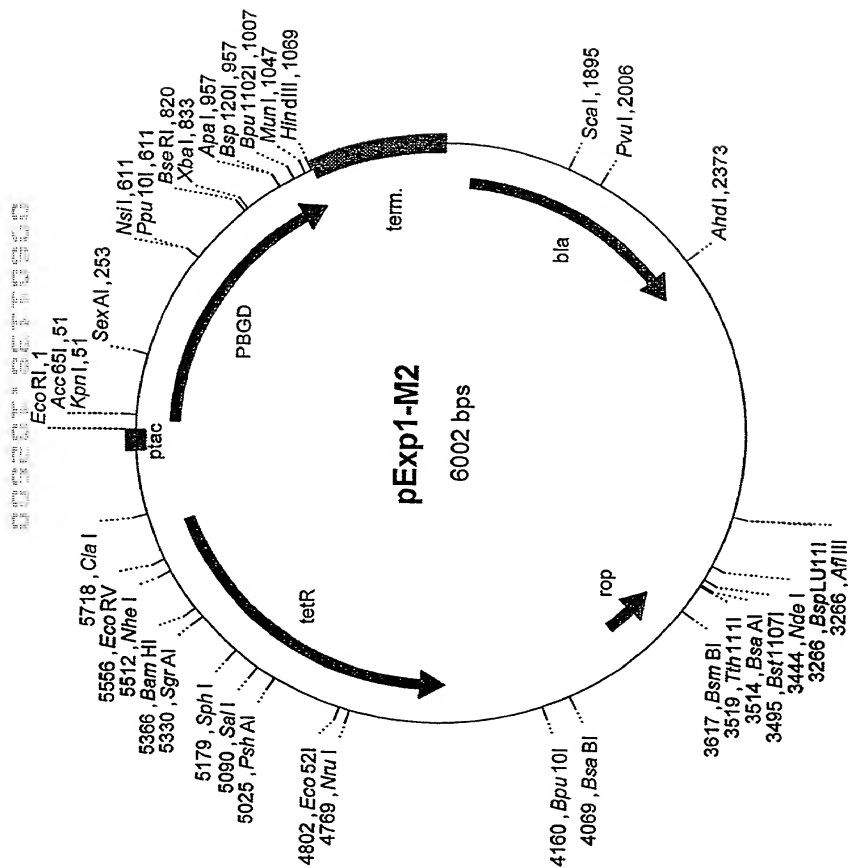


Fig. 7



8/31

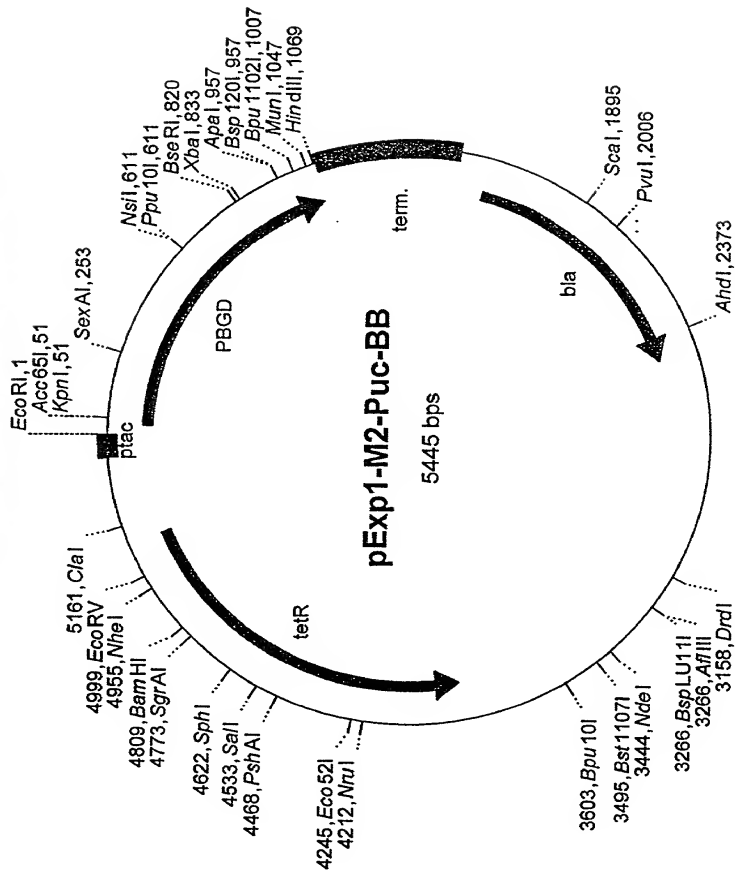


Fig. 8

9/31

## PBGD clone #1.1 in pBluescript SK- Sequence

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
CACCTGACGC	GGCCTGTAGC	GGCGCAITAA	GCGCGGCGGG	IGTGGTGGTT	ACGGCGAGCG
GTGGACTGCG	CGGGACATCG	CCGCGTAATT	GCGCGCGCCC	ACACCACCAA	TGCGCGTCGC
60					
IGACCGGTAC	ACTTGGCAGC	GCCCTAGCGC	CCGCTCCTTT	CGCTTCTTC	CCTTCCTTTC
ACTGGCGATG	TGAACGGTCG	CGGGATCGCG	GCGGAGGAAA	GCGAAAGAG	GGAAGGAAAG
120					
TCGCCACGTT	GCGCGGCTTT	CCCCGTCAAG	CTCTAAATCG	GGGGCTCCCT	TTAGGGTTCC
AGCGGTGCAA	GCGGCCGAAA	GGGGCAGTTC	GAGATTTAGC	CCCCGAGGGA	AATCCCAAGG
180					

NaeI  
 NgoMI  
 ▼

Fig. 9a

10/31

240  
GATTTAGTGC TTTACGGCAC CTCGACCCCA AAAAAGTTGA TTAGGTGAT GGTTCACGTA  
CTAAATCAG AAATGCCGTG GAGCTGGGT TTTTGAAGT AATCCCACTA CCAAGTGCA

300  
GTGGGCCATC GCCCTGATAG ACGGTTTTTC GCCCTTTGAC GTTGGAGTCC ACGTTCTTGA  
CACCCGTTAG CGGGACTATC TGCCAAAAG CGGGAAGCTG CAACCTCAGG TGCAAGAAAT

360  
ATAGTGGACT CTGTTCCTCA ACTGGAACAA CACTCAACCC TATCTCGGTC TATTCCTTTG  
TATCACCTGA GAACAAGTT TGACCTTGTT GTGAGTTGGG ATAGAGCCAG ATAAGAAAAC

Fig. 9b

11/31

10 20 30 40 50 60  
 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

---

420  
 ATTTATAAGG GATTTTGGCG ATTTGGGCTT ATTTGTTTAA AAATGAGCTG ATTTAACAAA  
 TAAATATTCC CTAAACGGC TAAAGCCGA TAACCAATTT TTACTCGAC TAAATGTTT

SspI  
 480  
 AATTTAAGC GAATTTTAAAC AAAATATTTA CGCTTACAAT TTCCATTGCG CATTGAGGCT  
 TTAAATTGCG CTTAAATTTG TTTTATAATT GCGAATGTTA AAGGTAAGCG GTAAGTCCGA

FspI  
 PvuI  
 540  
 GCGCACTGT TGGGAAGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA  
 CGCGTTGACA ACCCTTCCCG CTAGCCACGC CCGGAGAAGC GATAATGCGG TCGACCGCTT

Fig. 9c

12/31

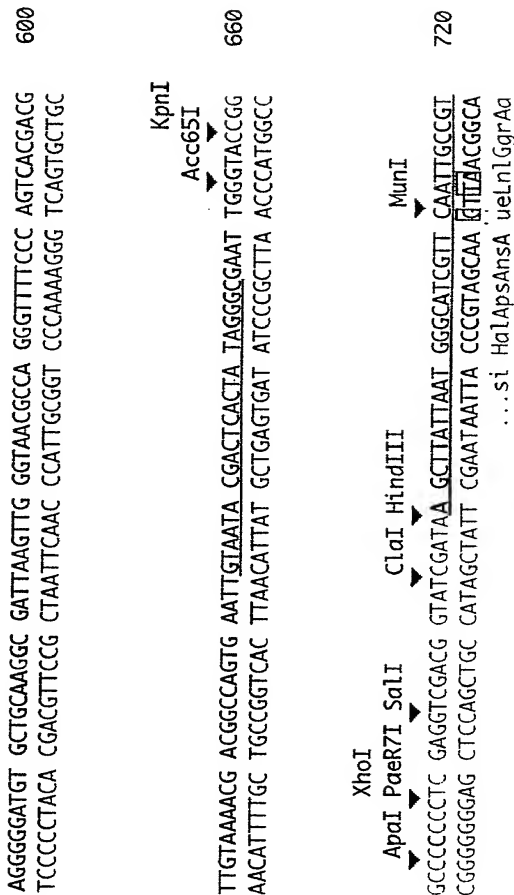


Fig. 9d

13/31

10 20 30 40 50 60  
 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

---

GCAACAICCA GGATGTTTT GGCTCCTTTG CTCAGCAACA AGTTGGCCAG GCTGATGCCC 780  
 CGTTGTAGGT CCTACAAAA CCGAGGAAC GAGTCGTTGT TCAACCGGTC CGACTACGGG  
 lAlaVpsAue LeInsAsyl aAlAylGsylr eSueLueLue LnsAalAueL reSeliYlGu

MscI  
▼

AAGTTCTGGG CAGCCAACTG GGGCCCTCGT GGAATGTTAC GAGCAGTGAT GCCTACCAAC 840  
 TTCAAGACCC GTCGGTTGAC CCCGGGAGCA CCTTACAATG CTCGTCACTA CGGATGGTTG  
 eLnsAnlGal AalAueLnlg orPylGgrAo rPelInsAgr AalArhTelI ylgLaVueln

ApoI  
▼

TGTGGTGCAT CCTCAGGGCC ATCTTCATGC TGGCAGGGA CATGGATGGT AGCCTGCGATG 900  
 ACACCCAGTA GGAGTCCCGG TAGAAGTACG ACCCGTCCCT GTACCTACCA TCGGACGTAC  
 lGorPpsAps AulGorPylG psAulGsiHn lGalaorPla VsiHelIrht aAnlGteMr

Fig. 9e

14/31

BsaI                      XbaI  
 ▼                                  ▼  
 GTCTCTTGTA TGCTATCTGA GCCGTCTAGA CTCCAGACTC CTCCAGTCAG GTACAGTTGC 960  
 CAGAGAACAT ACGATAGACT CGGCAGATCT GAGGTCCTGAG GAGGTCAGTC CATGTCAACG  
 hTulGnlGel IreSpsAreS yLGpsAueLr eSprTlavyl GylGrhTuel ryTuelnlgY  
  
 CCATCCTTCA TAGCTGTATG CACGGCTACT GGCACACTGC AGCCTCCTTC CAGGTGCCTC 1020  
 GGTAGGAAGT ATCGACATAC GTGCCGATGA CCGTGTGAGG TCGGAGGAAG GTCCACGGAG  
 lGpsAsyLte MaIArhTsiH laVaIalaVo rPlavresSy CylGylGulG uelSiHgrAu  
  
 AGGAAGGCCC TTTCAGCGAT GCAGCGAAGC AGAGTCTCGG GATCTGCGAG CACACCCACC 1080  
 TCCTTCGGGG AAAGTCGCTA CGTCGCTTCG TCTCAGAGCC CTAGCACGTÇ GTGTGGGTGG  
 elehPalAgr AulGalAelI syCgrAueLu eLrhTulGor PpsAsiHuel lavylGlaVu

Fig. 9f

15/31

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
AGATCCAAGA	TGTCCTGGTC	CTTGCTCGC	ACTTCCAGC	CCAAGGCCCC	CTGGCCCCACA
TCTAGGTCT	ACAGGACCAG	GAACCGAGCG	TGAAGGTGCG	GGTTCCGGGG	GACCGGGTGT
elpsAueLeI	IpsAnIgpsA	syLaIaIgrAl	aVulGIaVyl	GueLaIaYlG	nIgyLGlaVa
1140					
GCATACATGC	ATTCTCTCAGG	GTGCAGGATC	TGCCCAACCC	GGTTGTGCCA	GCCCATGCCG
CGTATGTACG	TAAGGAGTCC	CACGTCCTAG	ACGGGTTGGG	CCAACACCGT	CGGGTACGCG
lAryTteMsy	CulGulGorP	siHueLeIIn	lgyLGlaVgr	AnsAsiHprT	ylGteMgRAn
1200					
TGCAGGCCAG	CTGTTGCCAG	GATGATGGCA	CTGAATCTCT	GCTGCTCGTC	CAGCTTCCGA
ACGTCCGGTC	GACAACGCTC	CTACTACCGT	GACTTGAGGA	CGACGAGCAG	GTGGAAGGCT
lGueLylGal	ArhTalaueI	elielIalAr	eSehPulGnl	GnlGulGpsA	ueLsylgrAu
1260					

NsiI

BsmI



Fig. 9g



16/31

AGCGGGTGT TGAGTTTCC CCGAATACTC CTGAATCCA GATGCGGGAA CTTTCTCTGC 1320  
 TCGGCCACACA ACTCCAAAGG GGCTTATGAG GACTTGAGGT CTAGGCCCTT GAAAGAGACG  
 eLgrArhTns AueLnsAy1G grAelIreSg rAehPulGue LsiHorPehP syLgrAnlGu  
  
 AGCTGGGCTG CTCTTCGCAG GGAGCTGGT CCCACCACAC TCTTCTCTGG CAGGGTTTCT 1380  
 TCGACCCGAC GAGAAGCGTC CCTCGACCAA GGGTGGTGTG AGAAGAGACC GTCCCAAAGA  
 eLnlGatAal AgrAgrAueL reSreShhTy lGlaVlaVre SsyLulGorP ueLrhTulGu  
  
 AGGTCTTCC CAACAAATTT TGGGTGAAAG ACAACAGCAT CATGAGGGT TTCCCGCTTG 1440  
 TCCAGAAAGG GTTGTTTAAA ACCCACTTC TGTGTGTA GACTCCCAA AAGGGCGAAC  
 eLrhTsyLy1 GlaVehPsyL orPsiHehPl aVlaValAps AsiHorPnsA ulGgrAsyLs

BbsI  
▼

Fig. 9h

17/31

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
<hr/>					
BspMI					
▼					
CAGATGGCTC	CGATGGTGAA	GCCAGGAGGA	AGCACAGTGG	GCAGGTCCTT	CAAGGAGTGA
GTCTACCGAG	GCTACCACTT	CGGTCTCTCT	TCGTGTCACC	CGTCCAGGAA	GTTCTCTCACT
yCellalAyl	GelIrhTehP	ylGorPorPu	ellavrhTor	PuelpsAsyl	uelreSsiHl
					1500
ACAACAGGT	CCATTTCAAT	CTTCTCCAGG	GCATGTTCAA	GCTCCTTGGT	AAACAGGCTT
TGTTGGTCCA	GGTGAAGTAA	GAAGAGGTCC	CGTACAAGTT	CGAGGAACCA	TTTGTCCGAA
avlaVuelps	AlaVulGnsA	sylulGueLa	lAsiHulGue	LulGsyLrhT	ehPuelreSs
					1560
TTCTCTCCAA	TCTTAGAGAG	TGCAGTATCA	AGAATCTTGT	CCCTGTGGT	GGACATAGCA
AAGAGAGGTT	AGAATCTCTC	ACGTCATAGT	TCTTAGAACA	GGGACACCCA	CCTGTATCGT
yLulGylGel	IsylreSuel	alArhTpsAu	eLeIIsylPs	AylGrhTrhT	reSteMalae
					1620

Fig. 9i

18/31

1680  
 ATGATTTCAA ACTGCAGGCC AGGTACGAG GCTTTCATG TTGCCACCAC ACTGTCCTGC  
 TACTAAAGTT TGACGTCCGG TCCCATGCTC CGAAAGTTAC AACGTGGTG TGACAGGCAG  
 lIeIuIgeH PhlGueLyIG onPryTreSa lAsylueLrh TalAlaVlaV reSpsArhTn  
 KpnI  
 Acc65I  
 EcoRI  
 1740  
 TGTATGCCAG CAAGCTGGCT CTTGCGGGA CCCACGCCAA TCACTCTCAT GAATTCCTGC  
 ACATACGCTC GTTCGACCGA GAAGGCCCAT GGGTGGCTT AGTGAAGTA CTTAAGGACG  
 lGelIgrAal AueNlGreS sylgrArhTy lGlaVgrAel lIaVgrAtem  
 SmaI  
 XmaI  
 NotI  
 BamHI  
 SpeI  
 XbaI  
 EagI  
 SacI  
 Ecl136II  
 1800  
 AGCCCGGGG ATCCACTAGT TCTAGAGCGG CCGCCACCGC GGTGGAGCTC CAGCTTTTGT  
 TCGGGCCCC TAGGTGATCA AGATCTCGCC GCGGTGGCG CCACCTCGAG GTGAAACA

Fig. 9j

19/31

10 20 30 40 50 60  
1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

---

TCCCTTTAGT GAGGGTTAAT TTCGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG 1860  
AGGGAAATCA CTCCCAATTA AAGCTCGAAC CGCATTAGTA CCAGTATCGA CAAAGGACAC

TGAAATTGTT ATCGCTCAC AATTCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAG 1920  
ACTTTAACAA TAGCGGAGTG TTAAGGTGTG TTGTATGCTC GGCCTTCGTA TTTCACATTT

GCCTGGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGGGCTC ACTGCCCGCT 1980  
CGGACCCAC GGATTACTCA CTCGATTGAG TGTAATTAAC GCAACGGGAG TGACGGGGCGA

Fig. 9k

20/31

2040

TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAAATGAA TCGGCCAACG CGCGGGGAGA  
AAGGTCAGCC CTTTGGACAG CACGGTCGAC GTAATTACTT AGCCGGTTGC GCGCCCCCTCT

2100

GGCGGTTTGC GTATTGGGG CTCTTCCGCT TCCTCGGTCA CTGACTCGCT GCGCTCGGTC  
CCGCCAACG CATAACCCGC GAGAGGCCGA AGGAGCGAGT GACTGAGCGA CGCGAGCCAG

2160

GTTGCGCTGC GGCAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA  
CAAGCCGACG CCGCTCGCCA TAGTCAGTG AGTTCGCC ATATGCCAA TAGTGICIT

Fig. 9I

21/31

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
<hr/>					
TCAGGGGATA	ACGCAGGAAA	GAACATGTGA	GCAAAAGGCC	AGCAAAAGGC	CAGGAACCGT
AGTCCCCTAT	TGGTCCCTTT	CTTGACACT	CGTTTTCCGG	TCGTTTTCCG	GTCTTGGCA
<hr/>					
AAAAAGGCCG	CGTTGCTGGC	GTTTTTCCAT	AGGCTCCGCC	CCCTGACGA	GCATCACAAA
TTTTTCGGC	GCAACGACCG	CAAAAGGTA	TCCGAGGCGG	GGGACTGCT	CGTAGTGTTT
<hr/>					
AATCAGCGT	CAAGTCAGAG	GTGGGAAAC	CCGACAGGAC	TATAAAGATA	CCAGGCGTTT
ITAGCTGCGA	GTTCAGTCTC	CACCGCTTIG	GGCTGTCTTG	ATATTTCTAT	GGTCCGCAAA
<hr/>					

Fig. 9m

22/31

2400

CCCCCTGGAA GCTCCCTCGT GGGCTCTCT GTTCCGACCC TCCCGCTTAC CGGATACCTG  
GGGGGACCTT CGAGGGAGCA CGCGAGGGA CAAAGGCTGGG ACGGCGAATG GCCTATGGAC

2460

TCCGCTTTTC TCCCTTGGG AAGCGTGGG CTTTCTCATA GCTCAGCTG TAGGTATCTC  
AGGCGGAAAG AGGGAAGCCC TTCCACCCG GAAAGATAT CGAGTGGAC ATCCATAGAG

ApoI

2520

AGTTCGGTG AGGTCGTTGG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC  
TCAAGCCACA TCCAGCAAGC GAGGTTGAC CCGACACACG TGCTTGGGGG GCAAGTCGGG

Fig. 9n

23/31

10 20 30 40 50 60  
1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

GACCGCTGG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA- 2580  
CTGGCGACGC GGAATAGGCC ATTGATAGCA GAACTCAGGT TGGGCCATTCTGTGCTGAAT

TCGCCACTGG CAGCAGCCAC TGTAAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT 2640  
AGCGGTGACC GTGTCGGTG ACCATTGTCC TAACTGCTCTC GTCCCATACA TCCGCCACGA

ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTGGTATC 2700  
TGCTCAAGA ACTTCACCAC CGGATTGATG CCGATGTGAT CTTCCTGTCA TAAACCATAG

Fig. 90



24/31

2760

TGGCTCTGC TGAAGCCAGT TACCTTCGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA  
ACGCGAGACG ACTTCGGTCA ATGAAGCCT TTTTCTCAAC CATCGAGAAC TAGGCCGTTT

2820

CAAAACCACG CTGGTAGCGG TGGTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA  
GTTTGGTGGC GACCATCGCC ACCAAAAAA CAAAGTTCTG TCGTCTAATG CCGGTCTTTT

2880

AAAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGT CTGACGCTCA GTGGAACGAA  
TTTCCTAGAG TTCTTCTAGG AACTAGAAA AGATGCCCA GACTCGAGT CACCTTGCTT

Fig. 9p

25/31

10 20 30 40 50 60  
1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

AACACAGTT AAGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT- 2940  
TTGAGTGCAA TTCCTAAAA CCAGTACTCT AATAGTTTT CCTAGAAGTG GATCTAGGAA

TTAAATAAA AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC 3000  
AATTTAATTT TTACTTCAAA ATTTAGTTAG ATTTTCATATA TACTCAITTTG AACCAACTG

AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC 3060  
 TCAATGGTTA CGAATTAGTC ACTCCGTGGA TAGAGTCGCT AGACAGATAA AGCAAGTAGG  
 prTsi HsylelIueI reSalAylGe lIulGalAel InlGgrAnsA grAulGpsAt

Fig. 9q

26/31

ATAGTTGCCT GACTCCCCGT CGGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC 3120  
 TATCAACGGA CTGAGGGGCA GCACATCTAT TGATGCTATG CCCTCCCGAA TGGTAGACCG  
 eMrhTalAnl GreSylGrhT rhTryTelIl aVlaVelIgr AreSorPsyl ylgpsAorPy

BsaI  
▼

CCCAGTGTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCAGATTT ATCAGCAATA 3180  
 GGGTCACGAC GTTACTATGG CGCTCTGGGT GCGAGTGGCC GAGGTCTAAA TAGTCGTTAT  
 lGuelAlAal AeIleIyIG grAreSylGg rAulGylGal AylGreSsyl psAaIAeIle

AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACCTTTATC CGCTCCATC 3240  
 TTGGTCGGTC GGCCTTCCCG GCTCGGTCT TCACCAGGAC GTTGAAATAG GCGGAGGTAG  
 hPprTyIGal AorPueLala reSgrAueLu eLorPyIGal AlaVsyLpsA 'aIAulgteMp

Fig. 9r

27/31

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
CAGTCTATTA	ATTGTTGCCG	GGAAGCTAGA	GTAAGTAGTT	CGCCAGTTAA	TAGTTTGC
GTCAGATAAT	TAAACAACGGC	CCTTCGATCT	CATTTCATCA	GCGGTCAATT	ATCAAAACGCG
rTpsAelIue	LnlGnlGgrA	reSalAuelr	hTuelLueLul	GylGrhTuel	uelSylgrAu
AACGTTGTTG	CCATTGCTAC	AGGCATCGTG	GTGTCAGCGT	CGTCGTTTGG	TATGGCTTCA
TTGCAACAAC	GGTAACGATG	TCCGTAGCAC	CACAGTGCGA	GCAGCAAACC	ATACCCGAAGT
eLrhTrhTal	AteMaLaLaV	orPteMrhTr	hTpsAgrAul	GpsAnsAorP	ellalAulGn
TTCAGTCCG	GTTCCCAACG	ATCAAGGCGA	GTTACATGAT	CCCCCATGTT	GTGCAAAAAA
AAGTCAGGC	CAAGGGTTGC	TAGTCCGCT	CAATGTACTA	GGGGGTACAA	CACGTTTTTT
sAuelLulGor	PulGprTgrA	psAuelGrAr	hTlaVsiHps	AylGtelMnsA	siHuelehPa

FspI



3300

3360

3420

Fig. 9s

28/31

GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTATCA 3480  
 CGCCAATCGA GGAAGCCAGG AGGCTAGCAA CAGTCTTCAT TCAACCGCGG TCACAATAGT  
 lArhTuelLul GsYLorPyIG yIGelIrhTr hTuelLueLue LnsAaIAaIA rhTnsApsAr  
 PvuI  
 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTGA TGCCATCCGT AAGATGCTTT 3540  
 GAGTACCAAT ACCGTCTGTA CGTATTAGA GAATGACAGT ACGGTAGGCA TTCTACGAAA  
 eSteMhhTel lAlAaIAreS syCueluLgG rAlaVrhTte MylGpsArhT ueLsiHsYLu  
 ScaI  
 TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAAT AGTGTATGCG GCGACCGAGT 3600  
 AGACACTGAC CACTCATGAG TTGGTTCAGT AAGACTCTTA TCACATAGCG. CGCTGGCTCA  
 lGrhTlaVor PreSryTulG laVuelpsAn sAnlGreSry TsiHelIgrA grAylGueln

Fig. 9t

29/31

10	20	30	40	50	60
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
<hr/>					
TGCTCTTGCC	CGGCGTCAAT	ACGGGATAAT	ACCGCGCCAC	ATAGCAGAAC	TTTAAAAAGTG- 3660
ACGAGAACGG	GCCGCAGTTA	TGCCCTATTA	TGGCGCGGTG	TATCGTCTTG	AAATTTTCAC
lGuLgnlGyl	GalApsAelI	grAreSueLl	aValAylGsy	CueLueLLaV	sylehPrhTr
CTCATCATTG	GAAACGTTIC	TTCGGGGCGA	AAACTCTCAA	GGATCTTACC	GCTGTTGAGA 3720
GAGTAGTAAC	CTTTTGCAAG	AAGCCCCGCT	TTTGAGAGTT	CCTAGAATGG	CGACAACTCT
eStektelMor	PehPgrAulG	ulGorPgrAe	hPreSulGue	lelIsylYlG	reSnsAuelP
ApalI ▼					
TCCAGTTCGA	TGTAACCCAC	TCGTGCACCC	AACTGATCTT	CAGCATCTTT	TACTTTCACC 3780
AGGTCAAGCT	ACATTGGGTG	AGCACGTGGG	TTGACTAGAA	GTCTAGAGAA	ATGAAAGTGG
sAuelulGel	IryTyIGlaV	grAaLAylGu	eLnlGpsAul	GalApsAsyl	laVsylLaVu

Fig. 9u

30/31

3840

AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGCGC  
 TCGCAAAGAC CCACTCGTTT TTGTCCTTCC GTTTTACGGC GTTTTTCCTCC TTATTCCTCCG  
 el.rhTulGor PsiHalAehP laVorPueLS yCehPalAa1 AehPehPorP elIueLalAl

SspI

3900

ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG  
 TGTGCCTTTA CAACTTATGA GTATGAGAAG GAAAAAGTTA TAATAACTTC GTAAATAGTC  
 aVgrAehPsi HnlGelIreS teM

3960

GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAATAGGG  
 CCAATAACAG AGTACTCGCC TAATATATAA CTTACATAAA TCTTTTATTT..TGTTATCCC

Fig. 9v

31/31

10 20 30 40 50 60  
1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

GTTCCGGCGA CATTTCCTCCG AAAAGTGC  
CAAGCGCGT GTAAAGGGC TTTTCACG

3988

Fig. 9x



# **COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural, names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

*Method for treating acute intermittent porphyria (AIP) and other porphyric diseases*

the specification of which:

- ☐ is attached hereto
- ☐ was filed on 27 July 2000 as:
- ☒ United States Application Serial No. \_\_\_\_\_
- ☐ PCT Application No. \_\_\_\_\_
- ☐ and was amended on \_\_\_\_\_ (if applicable)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and that I acknowledge the duty to disclose information which is material to examination of this application in accordance with 37 C.F.R. § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119, 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S. listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

<u>Country (or PCT)</u>	<u>Application Number:</u>	<u>Filed (Day/Month/Year)</u>	Priority Claimed:	
			<u>Yes</u>	<u>No</u>
PCT	PCT/DK99/00040	27 January 1999		x
Danish	PA 1998 00112	27 January 1998	x	
Danish	PA 1998 01748	30 December 1998	x	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

<u>(Application Number)</u>	<u>(Filing Date)</u>
-----------------------------	----------------------

I hereby claim the benefit under 35 U.S.C. § 120 of any prior U.S. Application(s) or any prior PCT Application(s) designating the U.S. listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37

C.F.R., § 1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

Prior U.S. Application(s) [list additional applications on separate page]:

U.S. Serial No.: Filed (Day/Month/Year) PCT Application No. Status (patented, pending)

60/094,258 27 July 1998  
09/358,856 22 July 1999

provisor  
pending

I hereby appoint the following attorneys, with full power of substitution, association and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Sheridan Neimark 25,618	Reg. No. <u>20,520</u>	Roger L. Browdy	Reg. No.
Anne Mr. Kornbau 19,963	Reg. No. <u>25,884</u>	Norman J. Latker	Reg. No.
Iver P. Cooper 18,453	Reg. No. <u>28,005</u>	A. Fred Starobin	Reg. No.
Allen C. Yun 33,478*	Reg. No. <u>37,971*</u>	Nick S. Bromer	Reg. No.

\* Patent Agent


Address all Correspondence to:

Direct all telephone calls to:  
**BROWDY AND NEIMARK**  
(202) 628-5197

**BROWDY AND NEIMARK**  
419 Seventh Street, N.W., Suite 300  
Washington, D.C. 20004

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTORS:

Full name of first or sole inventor (capitalize surname)	Inventor's signature	Date
FOGH, Jens		
Residence (City, State & Country)	Citizen of	
Bjergagervej 37, DK-3400 Hillerød, Denmark	Denmark	
Post Office Address (include postal code, and country)		
Bjergagervej 37, DK-3400 Hillerød, Denmark		

20

Full name of additional inventor (capitalize surname)	Inventor's signature	Date
GELLERFORS, Pär		
Residence (City, State & Country)	Citizen of	
Lagmannsvägen 13, SE-18163 Lidingö, Sweden	Sweden	
Post Office Address (include postal code, and country)		
Lagmannsvägen 13, SE-18163 Lidingö, Sweden		
Full name of additional inventor (capitalize surname)	Inventor's signature	Date
Residence (City, State & Country)	Citizen of	
Post Office Address (include postal code, and country)		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING.  
 ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO  
 EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED.  
 ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

## SEQUENCE LISTING

534 Rec'd PCT/PT 27 JUL 2000

&lt;110&gt; HemeBiotech A/S

<120> New therapeutic method for treating patients with Acute  
Intermittent Porphyria (AIP) and other porphyric  
diseases

&lt;130&gt; Sequence

&lt;140&gt; -

&lt;141&gt; 1999-01-27

&lt;150&gt; 0112/98

&lt;151&gt; 1998-01-27

&lt;150&gt; PA 1998 01748

&lt;151&gt; 1998-12-30

&lt;160&gt; 12

&lt;170&gt; PatentIn Ver. 2.0

&lt;210&gt; 1

&lt;211&gt; 1035

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 1

```

atgagagtga ttccgctggg taccgcgaag agccagcttg ctgcataca gacggacagt 60
gtggtggcaa cattgaaagc ctcgaccct ggccctgcagt ttgaaatcat tgctatgtcc 120
accacagggy acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctggttgt tcactccttg 240
aaggacctgc ccactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300
aaccctcatg atgctgttgt ctttcaccca aaatttgttg ggaagaccct agaaaccctg 360
ccagagaaga gtgtgggtgg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420
ttcccgcate tggagtccag gagtattcgg ggaaacctca acaccggct tcggaagctg 480
gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcatgggc 540
tggcacaacc ggggtgggca gatcctgcac cctgaggaat gcatgtatgc tgtgggccag 600
ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttgatctt ggtgggtgtg 660
ctgcacgata ccgagactct gcttcgctgc atcctgaaa gggccttccct gaggcacctg 720
gaaggaggct cgagtgtgac agtagccgtg catacagcta tgaaggatgg gcaactgtac 780
ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggct 840
accatccatg tcctgcccc gcatgaagat ggccctgagg atgaccaca gttggtaggc 900
atcactgctc gtaacattcc acgagggccc cagttggctg ccagaaactt gggcatcagc 960
ctggccaact tgttgctgag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020
aacgatgccc attaa                                     1035

```

<210> 2  
 <211> 1035  
 <212> DNA  
 <213> Human tissue

<400> 2  
 atgagagtga ttccgcgtggg taccgcgaag agccagcttg ctcgcataca gacggacagt 60  
 gtgggtggcaa cattgaaagc ctcgtagcctt ggccctgcagt ttgaaatcat tgctatgtcc 120  
 accacaggggg acaagattctt tgatactgca ctctctaaga ttggagagaa aagcctgttt 180  
 accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctgggtgt tcaactccttg 240  
 aaggacctgc ccaactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300  
 aacctcatg atgctgttgt ctttcaccca aaatttggtg ggaagacctt agaaaccttg 360  
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420  
 ttcccgcatc tggagttcag gtagtattcg ggaacctca acacccggct tcggaagctg 480  
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcatgggc 540  
 tggcacaaacc ggggtgggca gatcctgcac cctgaggaat gcattgatgc tgtgggccag 600  
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatct ggtgggtgtg 660  
 ctgcacgac cagagactct gcttcgctgc atcgctgaaa gggccttctt gaggcacctg 720  
 gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780  
 ctgactggag gactctggag tctagacggc tcagatagca tacaagagac catgcaggct 840  
 accatccatg tcctctgcca gcatgaagat ggccctgagg atgacccaca gttggtaggc 900  
 atcaactgctc gtaacattcc acgagggccc cagttagctg ccagaaactt gggcatcagc 960  
 ctggccaact tgttgctgag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020  
 aacgatgccc attaa 1035

<210> 3  
 <211> 1035  
 <212> DNA  
 <213> Human tissue

<400> 3  
 atgagagtga ttccgcgtggg taccgcgaag agccagcttg ctcgcataca gacggacagt 60  
 gtgggtggcaa cattgaaagc ctcgtagcctt ggccctgcagt ttgaaatcat tgctatgtcc 120  
 accacaggggg acaagattctt tgatactgca ctctctaaga ttggagagaa aagcctgttt 180  
 accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctgggtgt tcaactccttg 240  
 aaggacctgc ccaactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300  
 aacctcatg atgctgttgt ctttcaccca aaatttggtg ggaagacctt agaaaccttg 360  
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420  
 ttcccgcatc tggagttcag gtagtattcg ggaacctca acacccggct tcggaagctg 480  
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcatgggc 540  
 tggcacaaacc ggggtgggca gatcctgcac cctgaggaat gcattgatgc tgtgggccag 600  
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatct ggtgggtgtg 660  
 ctgcacgac cagagactct gcttcgctgc atcgctgaaa gggccttctt gaggcacctg 720  
 gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780  
 ctgactggag gactctggag tctagacggc tcagatagca tacaagagac catgcaggct 840  
 accatccatg tcctctgcca gcatgaagat ggccctgagg atgacccaca gttggtaggc 900  
 atcaactgctc gtaacattcc acgagggccc cagttagctg ccagaaactt gggcatcagc 960  
 ctggccaact tgttgctgag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020

aacgatgccc attaa

1035

&lt;210&gt; 4

&lt;211&gt; 1034

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 4

```

atgagagtga ttcgcgtggg tacccgcaag agccagcttg ctgcataca gacggacagt 60
gtggtggcaa cattgaaagc ctcgtaacct ggctgcagt ttgaaatcat tgctatgtcc 120
accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctggttgt tcaactcctt 240
aaggacctgc ccaactgtgt tctcctctggc ttcaccatcg gagccatctg caagcgggaa 300
aaccctcatg atgctgttgt cttcacccaa aatttgttgg gaagacccta gaaacccctg 360
cagagaagag tgtggtggga accagctccc tgcgaagagc agcccagctg cagagaaagt 420
tcccgcatct ggagttcagg agtattcggg gaaacctcaa caccggctt cggaagctgg 480
acgagcagca ggagttcagt gccatcatcc tggcaacagc tggcctgcag cgcattgggt 540
ggcacaaccg ggtggggcag atcctgcacc ctgaggaatg catgtatgct gtgggccagg 600
gggccttggg cgtggaagtg cgagccaagg accaggacat cttggtatctg gtgggtgtgc 660
tgcacgatcc cgagactctg cttcgtctga tcgctgaaag ggcccttctg aggcacctgg 720
aaggaggctg cagtgtgcca gttagccgtg atacagctat gaaggatggg caactgtacc 780
tgactggagg agtctggagt ctgacggctc cagatagcat acaagagacc atgcaggcta 840
ccatccatgt ccctgccagc catgaagatg gccctgagga tgaccacagc ttggtaggca 900
tcaactgctg taacattcca cgagggcccc agttggctgc ccagaacttg ggcacagcc 960
tggccaactg gttgctgagc aaaggagcca aaaacatcct ggatgttgca cggcaattga 1020
acgatgccca ttaa                                     1034

```

&lt;210&gt; 5

&lt;211&gt; 1035

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 5

```

atgagagtga ttcgcgtggg tacccgcaag agccagcttg ctgcataca gacgggacgt 60
gtggtggcaa cattgaaagc ctcgtaacct ggctgcagt ttgaaatcat tgctatgtcc 120
accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctggttgt tcaactcctt 240
aaggacctgc ccaactgtgt tctcctctggc ttcaccatcg gagccatctg caagcgggaa 300
aaccctcatg atgctgttgt cttcacccaa aaatttgttg ggaagacctt agaaaccttg 360
ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaagg 420
ttcccgatc tggagttcag gagtattcgg ggaacctcaa acaccggct tcggaagctg 480
gacgagcagc aggagttcag tgtcatcatc ctggcaacag ctggcctgca gcgcatgggc 540
tggcacaacc gtgtgtggca gatcctgcac cctgaggaat gcatgtatgc tgtggggccag 600
ggggccttgg cgtggaagt gcgagccaag gaccaggaca tcttggtatc ggtgggtgtg 660
ctgcacgatc ccgagactct gcttcgtctg atcgtgaaa gggccttcct gaggcacctg 720
gaaggaggct gcagtggtcc agtagccctg catagagcta tgaaggatgg gcaactgtac 780
ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggct 840
accatccatg tccctgccca gcattgaagat ggccctgagg atgaccaca gttggtaggc 900

```

atcactgctc gtaacattcc acgagggccc cagttagctg ccagaactt gggcatcagc 960  
 ctggccaact tgttgcctgag caagggagcc aaaaacatcc tggatgttgc acggcaattg 1020  
 aacgatgccc attaa 1035

<210> 6

<211> 1035

<212> DNA

<213> Human tissue

<400> 6

atgagagtga ttgcgctggg taccgcgaag agccagcttg ctgcataca gacggacagt 60  
 gtgggtggcaa cattgaaagc ctcgtagcct ggcctgcagt ttgaaatcat tgctatgtcc 120  
 accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180  
 accaaggagc ttgaacatgc cctgggagaag aatgaagtgg acctggttgt tcaactccttg 240  
 aaggacctgc ccactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300  
 aaccctcatg atgctgttgt ctttcaccca aaatttgttg ggaagacctt agaaaccttg 360  
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420  
 ttcccgcatc tggagttcag gagtattcgg ggaaacctca acaccggct tcggaagctg 480  
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcatgggc 540  
 tggcacaacc ggggtgggca gatcctgcac cctgaggaat gcatgtatgc tgtgggcccag 600  
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatct ggtgggtgtg 660  
 ctgcacgac cagagactct gcttcgctgc atcgctgaaa gggccttctt gaggcacctg 720  
 gaaggaggtt gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780  
 ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggct 840  
 accatccatg tcctcgccca gcatgaagat ggccctgagg atgaccacca gttggtaggc 900  
 atcactgctc gtaacattcc acgagggccc cagttagctg ccagaactt gggcatcagc 960  
 ctggccaact tgttgcctgag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020  
 aacgatgccc attaa 1035

<210> 7

<211> 1034

<212> DNA

<213> Human tissue

<400> 7

atgagagtga ttgcgctggg taccgcgaag agccagcttg ctgcataca gacggacagt 60  
 gtgggtggcaa cattgaaagc ctcgtagcct ggcctgcagt ttgaaatcat tgctatgtcc 120  
 accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180  
 accaaggagc ttgaacatgc cctgggagaag aatgaagtgg acctggttgt tcaactccttg 240  
 aaggacctgc ccactgtgct tcctcctggc ttcaccatcg gagccatctg caagcgggaa 300  
 aaccctcatg atgctgttgt ctttcaccca aaatttgttg ggaagacctt agaaaccttg 360  
 ccagagaaga gtgtggtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420  
 ttcccgcatc tggagttcag gagtattcgg ggaaacctca acaccggct tcggaagctg 480  
 gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca gcgcatgggc 540  
 tggcacaacc ggggtgggca gatcctgcac cctgaggaat gcatgtatgc tgtgggcccag 600  
 ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggtatct ggtgggtgtg 660  
 ctgcacgact cagagactct gcttcgctgc atcgctgaaa gggccttctt gaggcacctg 720  
 gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg gcaactgtac 780

```

ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggct 840
accatccatg tccctgcccc gcatgaagat ggcctgagg atgaccacaca gttggtaggc 900
atcactgtct gtaacattcc acgagggccc cagttggctg cccagaactt gggcatcagc 960
ctggccaact tgttctgtag caaaggagcc aaaaacatcc tggatgttgc acggcaatta 1020
acgatgcccc ttaa                                     1034

```

&lt;210&gt; 8

&lt;211&gt; 1035

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 8

```

atgagagtga ttgcgtggg taccgcgaag agccagcttg ctgcataca gacggacagt 60
gtggtggcaa cattgaaagc ctcgtaacct ggcctgcagt ttgaaatcat tgctatgtcc 120
accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa aagcctgttt 180
accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctggttgt tcaactcctg 240
aaggacctgc ccactgtgct tctcctctggc ttaccatcg gagccatctg caagcgggaa 300
aaccctcatg atgctgttgt ctttcaccca aaatttggtg ggaagacctt agaaaacctg 360
ccagagaaga gtgtgtggg aaccagctcc ctgcgaagag cagcccagct gcagagaaag 420
ttcccgcac tggagttcag gagtattcgg ggaaacctca acaccggct tcggaagctg 480
gacgagcagc aggagttcag tgccatcac ctggcaacag ctggcctgca gcgcatgggc 540
tggcacaacc ggggtgggca gatcctgcac cctgaggaat gcatgtatgc tgtggggcag 600
ggggccttgc gctgtgaaat gcgagccaa gaccaggaca tcttgatct ggtgggtgtg 660
ctgcacgac cegagactct gcttcgctgc atcgctgaaa gggccttctt gaggcacctg 720
gaaggaggct gcagtgtgcc agtagccgtg catcacgcta tgaaggatgg gcaactgtac 780
ctgactggag gagtctggag tctagacggc tcagatagca tacaagagac catgcaggcc 840
accatccatg tccctaccca gcatgaagat ggcctgagg atgaccacaca gttggtaggc 900
atcactgtct gtaacattcc acgagggccc cagttggctg cccagaactt gggcatcagc 960
ctggccaact tgttctgtag caaaggagcc aaaaacatcc tggatgttgc acggcaattg 1020
aacgatgccc attaa                                     1035

```

&lt;210&gt; 9

&lt;211&gt; 3988

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 9

```

cacctgacgc gccctgtagc ggcgcattaa gcgcggcggg tgtggtggtt acgcgcagcg 60
tgaccgctac acttgccagc gccctagcgc ccgctccttt cgctttcttc ccttctcttc 120
tcgccacgtt cgccgctttt ccccgctcaag ctctaatacg ggggctccct ttagggttcc 180
gatttagtgc tttacggcac ctgcacccca aaaaacttga ttagggtgat ggttcacgta 240
gtggggccatc gccctgatag acggttttgc gccctttgac gttggagtc acgttcttta 300
atagtggact cttgttccaa actggaacaa cactcaacct tatctcggtc tattcttttg 360
atttataagg gattttgcgc atttcggcct attggttaaa aaatgagctg atttaacaaa 420
aatttaacgc gaattttaac aaaatattaa cgcttacaat ttccattcgc cattcaggct 480
gcgcaactgt tgggaagggc gatcggtgcg ggcctcttcg ctattacgcc agctggcgaa 540
agggggatgt gctgcaagc gattaagttg ggtaacgcca ggggtttccc agtcacgacg 600
ttgtaaaacg acggccagtg aattgtaata cgactcacta tagggcgaat tgggtaccgg 660

```



gccccccctc gaggtcgacg gtatcgataa gcttattaat gggcatcggt caattgccgt 720  
 gcaacatcca ggatgttttt ggctcctttg ctacagcaaca agttggccag gctgatgcc 780  
 aagttctggg cagccaactg gggccctcgt ggaatgttac gagcagtgat gcctaccaac 840  
 tgtgggtcat cctcagggcc atcttcatgc tgggcaggga catggatggt agcctgcatg 900  
 gtctcttgta tgctatctga gccgtctaga ctccagactc ctccagtcag gtacagttgc 960  
 ccatccttca tagctgtatg cacggctact ggcacactgc agcctcctc caggtgccctc 1020  
 aggaaggccc tttcagcagc gcagcgaagc agagctctcg gatcgtgcag cacaccacc 1080  
 agatccaaga tgcctgggtc cttggctcgc acttccacgc ccaaggcccc ctggcccaca 1140  
 gcatacatgc attcctcagg gtgcaggatc tgcccaaccc ggttgtgccca gccatgctgc 1200  
 tgcaggccag ctgttggccg gatgatggca ctgaactcct gctgctcgtc cagcttccga 1260  
 agccgggtgt tgagggttcc ccgaatactc ctgaactcca gatcgcgga ctttctctgc 1320  
 agctgggtg ctcttcgcag ggagctggtt ccaccacac tcttctctgg cagggtttct 1380  
 agggctcttc caacaaattt tgggtgaaag acaacagcat catgagggtt tcccgcgtg 1440  
 cagatggctc cgatggtgaa gccaggagga agcacagtg gcaggtcctt caaggagtga 1500  
 acaaccaggt ccacttcatt cttctccagg gcatgttcaa gctccttgg aaacaggctt 1560  
 ttctctccaa tcttagagag tgcagtatca agaactctgt cccctgtggg ggacatagca 1620  
 atgatttcaa actgcaggcc agggtagcag gctttcaatg ttgccaccac actgtccgtc 1680  
 tgtatgcgag caagctggct cttgggggta ccacgcgaa tcactctcat gaattcctgc 1740  
 agccggggg atccactagt tctagagcgg ccgccaccgc ggtggagctc cagcttttgt 1800  
 tccttttagt gagggttaat ttcgagcttg gcgtaatcat ggtcatagct gttcctgtg 1860  
 tgaaattgtt atccgctcac aattccacac aacatacgag ccggaagcat aaagtgtaaa 1920  
 gctgggggtg cctaagtagt gagtaactc acattaattg cgttgcgctc actgcccgct 1980  
 ttccagtctg gaacactgtc gtgccagctg cattaatgaa tcggccaacg cgcggggaga 2040  
 ggcggtttgc gttattgggg ctcttccgct tctcgtctca ctgactcgct gcgctcggtc 2100  
 gttcgagctg ggcgagcgtt atcagctcac tcaaaggcgg taactagttt atccacagaa 2160  
 tcaggggata acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc caggaaacct 2220  
 aaaaaggccg cgttgctggc gtttttccat aggctccgcc cccctgacga gcatcaciaa 2280  
 aatcgacgct caagtacagag gtggcgaaac ccgacaggac tataaagata ccaggcgctt 2340  
 cccctggaaa gctccctcgt gcgctctcct gttccgacc tgcgccttac cggataacct 2400  
 tccgcttttc cctcttcggg aagcgtggcg ctttctcata gctcacgctg taggtatctc 2460  
 agttcggtgt aggtcgttgc ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc 2520  
 gaccgctcgc ccttatccgg taactatcgt cttgagtcga acccggttag acacgactta 2580  
 tcgccactg cagcagccac tggtaacagc attagcagag cgaggtatgt agcggtgctg 2640  
 acagagttct tgaagttgtg gccaaactac ggctacacta gaagacagat atttggatc 2700  
 tgcgctctgc tgaagccagt taccttcgga aaaagagttg gtatgctctg atccggcaaa 2760  
 caaaccaccg ctggtagcgg tgggtttttt gtttgcaagc agcagattac gcgcagaaaa 2820  
 aaaggatctc aagaagatcc tttgatcttt tctacggggt ctgacgctca gtggaacgaa 2880  
 aactcagttt aagggtattt ggtcatgaga ttatcaaaaa ggaacttctac ctatgactct 2940  
 ttaaatataa aatgaagttt taaatcaatc taaagtatat atgagtaaac ttggtctgac 3000  
 agttaccaat gcttaatcag tgaggcacct atctcagcga tctgtctatt tctgtcatcc 3060  
 atagttgcct gactccccgt cgtgtagata actacgatac gggagggtctt accatctggc 3120  
 cccagtgtct caatgatacc gcgagaccga cgctcaccgg ctccagattt atcagcaata 3180  
 aaccagccag ccggaagggc cagcgcagag agtggctcgt caactttatc cgcctctcatc 3240  
 cagtctatta attgttgcg ggaagctaga gtaagtagtt ccgcagttaa tagtttgcgc 3300  
 aacgttgttg ccatgtctac aggcactcgt gtgtcacgct cgtcgtttgg tatggcttca 3360  
 ttcagctccg gttcccaacg atcaaggcga gttacatgat ccccatgatt gtgcaaaaaa 3420  
 gcggttagct ccttcggtcc tccgactcgt gtcagaagta agttggccgc agtgttatca 3480  
 tctatgggta tggcagcact gcataattct cttactgtca tgccatccgt aagatgcttt 3540

```

tctgtgactg gtgagtactc aaccaagtc a tcttgagaat agtgtatgcg ggcaccgagt 3600
tgctcttgcc cggcgctcaat acgggataat accgcgccac atagcagaac tttaaaagtg 3660
ctcatcattg gaaaacgttc ttccggggcga aaactctcaa ggatcttacc gctgttgaga 3720
tccagttcga tgtaaccacc tcgtgcaccc aactgatctt cagcatcttt tactttcacc 3780
agcgtttctg ggtgagcaaa aacaggaagg caaaatgccg caaaaaaggg aataaggggc 3840
acacggaaat gttgaatact catactcttc ctttttcaat attattgaag catttatcag 3900
ggttattgtc tcattgagcg atacataatt gaattgtatt agaaaaataa acaaataggg 3960
gttccgcgca catttccccg aaaagtgc 3988

```

&lt;210&gt; 10

&lt;211&gt; 1260

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 10

```

cacaggaaac agctatgacc atgattacgc caagctcgaa attaacccct actaaagggg 60
acaaaagctg gagctccacc gcggtggcgg cogctctaga actagtggat cccccgggct 120
gcaggaaatt atgagagtga ttccgctggg tacccgcaag agccagcttg ctgcataca 180
gacggacagt gtggtggcaa cattgaaagc ctcgtaaccct ggctgcagt ttgaaatcat 240
tgctatgtcc accacagggg acaagattct tgatactgca ctctctaaga ttggagagaa 300
aagcctgttt accaaggagc ttgaacatgc cctggagaag aatgaagtgg acctggttgt 360
tactccttg aaggacctgc ccaactgtgt tccctctggc ttcaccatcg gagccatctg 420
caagcgggaa aaccctcatg atgctgttgt ctttaccaca aaatttgttg ggaagaccct 480
agaaacctg ccagagaaga gtgtgtgtgg aaccagctcc ctgcgaagag cagccagct 540
cagagagaag ttcccgcac ttggagttcag gagtattcgg ggaaacctca acaccggct 600
tcggaagctg gacgagcagc aggagttcag tgccatcatc ctggcaacag ctggcctgca 660
gcgcatggcg tggcacaacc gggttgggca gatcctgcac cctgaggaat gcatgtatgc 720
tgtggggcag ggggccttgg gcgtggaagt gcgagccaag gaccaggaca tcttggatct 780
ggtgggtgtg ctgcacgac ccgagactct gcttcgctgc atcgtcgaaa gggccttcc 840
gaggcacctg gaaggaggct gcagtgtgcc agtagccgtg catacagcta tgaaggatgg 900
gcaactgtac ctgactggag gactctggag tctagacggc tcagatagca tacaagagac 960
catgcaggct accatccatg tccctgcccc gcatgaagat ggccctgagg atgaccaca 1020
gttggtagcg atcactgtcc gtaacattcc acgagggcc cagttggctg ccagaaact 1080
gggcatcagc ctggccaact gttgctgag caaaggagcc aaaaacatcc gctgtgttgc 1140
acggcaattg aacgatgccc attaataagc ttatcgatac cgtcgacctc gagggggggc 1200
ccggtaccca attcgcccta tagtgagtcg tattacaatt cactggccgt cgttttcaaa 1260

```

&lt;210&gt; 11

&lt;211&gt; 5445

&lt;212&gt; DNA

&lt;213&gt; Human tissue

&lt;400&gt; 11

```

gaattctaac ataagttaag gaggaaaaaa aaatgagagt tattcgtgtc ggtacccgca 60
agagccagct tgctcgcata cagacggaca gtgtggtggc aacattgaaa gcctcgtaac 120
ctggcctgca gtttgaatc attgctatgt ccaccacagg ggacaagatt cttgatactg 180
cactctctaa gattggagag aaaagcctgt ttaccaagga gcttgaacat gcctggaga 240
agaatgaagt ggacctggtt gttcactcct tgaaggacct gccactgtg ctctcctcgt 300

```

gcttcaccat cgagaccatc tgcaagcggg aaaaccctca tgatgctgtt gtctttcacc 360  
 caaaatttgt tgggaagacc ctagaaaccc tggcagagaa gagtgtgggt ggaaccagct 420  
 ccctgcgaag agcagccag ctgcagagaa agttcccga tctggagttc aggagtatcc 480  
 ggggaaacct caacaccggg cttcggaagc tggacagaca gcaggagttc agtgcacatca 540  
 tctctggcaac agctggcctg cagcgcattg gctggcaca cggggttggg cagatccctgc 600  
 accctgagga atgcatgtat gctgtgggct agggggcctt gggcggtgaa gtgcgagaca 660  
 aggaccagga catcttggat ctggtgggtg tgcgtgcaca tcccagact ctgcttcgct 720  
 gcatcgctga aagggccttc ctgaggcacc tgggaaggagg ctgcagtgtg ccagtagaccg 780  
 tgcatacagc tatgaaggat gggcaactgt acctgactgg aggagtctgg agtctagaccg 840  
 gctcagatag catacaagag accatgcagg ctaccatcca tgcctctgcc cagcatgaag 900  
 atggccctga ggatgacca cagttggtag gcatcactgc tcgtaacatt ccacgagggc 960  
 cccagttggc tgcccagaac ttgggcatca gcctggccaa cttgtgtgct agcaaaggag 1020  
 ccaaaaacat cctggatgtt gcacggcaat tgaacgatgc ccattaataa gcttctgttt 1080  
 ttggcggatga gagaagattt tcagcctgat acagattaaa tcagaacgca gaagcgggtct 1140  
 gataaaacag aatttgcctg gcgcgcatg cgcggtgtgc ccactgcacc ccatgccgaa 1200  
 ctcagaagtg aaacgcgcta gcgcgcatgg tagtgtgggg tctccccatg cgagagtagg 1260  
 gaactgccag gcatcaaata aaacgaaagg ctacgtcgaa agactggggc tttcgtttta 1320  
 tctgtttgtt gtcggtgaac gctctcctga gttagacaaa tccgcccggga gcggatttga 1380  
 acgttgcgaa gcaacggccc ggaggggtgg gggcaggacg cccgccataa actgccaggc 1440  
 atcaaatata gcagaaggcc atcctgacgg atggcctttt tgcgtttcta caaactcttt 1500  
 tgtttatttt tctaataata ttcaaatatg tatccgtca tgagacaata accctgataa 1560  
 atgcttcaat aatattgaaa aaggaaagat atgagtatcc aacatttccg tgcgcctt 1620  
 attccctttt ttggcggcat ttgccttccct gtttttgctc accagaaac gctggtgaaa 1680  
 atcaaaatga ctgaagataa gttgggtgca cgagtgggtt acatcgcaat ggcattcaac 1740  
 agcggtaaga tccttgagag ttttcgcccc gaagaacgct ttccaatgat gagcactttt 1800  
 aaagtctctg tatgtggcgc ggtattatcc cgtgttgacg cggggcaaga gcaactcggg 1860  
 cgccgcatac actattctca gaatgacttg gttgagtact caccagtcac agaaaagcat 1920  
 cttaacggat gcatgacact aagagaatta tgcagtgtcg ccataaccat gagtgtataa 1980  
 actgcggcca acttacttct gacaacgac ggaggaccga aggagctaac cgcttttttg 2040  
 cacaacatgg gggatcatgt aactcgcctt gatcgttggg aaccggagct gaatgaagcc 2100  
 ataccaaacg acgagcgtga caccacgatg cctgtagcaa tggcaacaac gttgcgcaaa 2160  
 ctattaactg gcgaactact tactctagct tcccggcaac aattaataga ctggatggag 2220  
 gcggataaag ttgcaggacc acttctgcgc tcggcccttc cggctggctg gtttattgct 2280  
 gataaatctg gagccggtga gcgtgggtct cgcggtatca ttgcagcact ggggccagat 2340  
 ggtaagccct ccggtatcgt agttatctac acgacgggga gtcaggcaac tatggtatga 2400  
 cgaaatagac agatcgctga gataggtgcc tcaactgatta agcattggta actgtcagac 2460  
 caagtttact catataacta ttgattgat ttaaaacttc atttttaatt taaaaggata 2520  
 taggtgaaga tcctttttga taatctcatg accaaaatcc cttaacgtga gttttcgttc 2580  
 cactgagcgt cagaccccgat agaaaagatc aaaggatctt cttgagatcc tttttttctg 2640  
 gcgctaattc gctgcttgca aacaaaaaaa ccaccgctac cagcggttgt tggtttgcg 2700  
 gatcaagagc taccaactct ttttccgaag gtaactggct tcagcagagc gcagatacca 2760  
 aatactgtcc tttctagtga gccgtagtta gccaccact tcaagaactc ttagcaccg 2820  
 cctacatacc tcgctctgct aatcctgtta ccagtggctg ctgccagtgg cgataagtcg 2880  
 tgtcttaccg ggttggactc aagacgatag ttaccggata aggcgcagcg gtcgggctga 2940  
 acgggggggt cgtgcacaca gccacgcttg gagcgaacga cctacaccga actgagatca 3000  
 ctacagcgtg agctatgaga aagcggcacg cttcccgaag ggagcaaggc gacaggtat 3060  
 ccggtaagcg gcagggtcgg aacaggagag cgcacgaggg agcttcagg gggaaacgcc 3120  
 tggtatcttt atagtctctg cgggtttcgc cacctctgac ttgagcgtcg atttttgtga 3180

```

gaaatcattg ctatgtccac cacaggggac aagattcttg atactgcact ctctaagatt 240
ggagagaaaa gcctgtttac caaggagctt gaacatgccc tggagaagaa tgaagtggac 300
ctggttgttc actccttgaa ggacctgccc actgtgcttc ctccctggctt caccatcgga 360
gccatctgca agcgggaaaa ccctcatgat gctgttgtct ttcacccaaa atttgttggg 420
aagaccctag aaaccctgcc agagaagagt gtggtgggaa ccagctccct gcgaagagca 480
gcccagctgc agagaaaagtt cccgcatctg gagttcagga gtattcgggg aaacctcaac 540
acccggcttc ggaagctgga cgagcagcag gagttcagtg ccatcatcct ggcaacagct 600
ggcctgcagc gcatgggctg gcacaaccgg gttgggcaga tctgcaccc tgaggaatgc 660
atgtatgctg tgggccaggg ggccttgggc gtggaagtgc gagccaagga ccaggacatc 720
ttggatctgg tgggtgtgct gcacgatccc gagactctgc ttcgctgcat cgctgaaagg 780
gccttcctga ggcacctgga aggaggctgc agtgtgccag tagccgtgca tacagctatg 840
aaggatgggc aactgtacct gactggagga gtctggagtc tagacggctc agatagcata 900
caagagacca tgcaggctac catccatgtc cctgcccagc atgaagatgg ccctgaggat 960
gaccacagtg tggtaggcat cactgctcgt aacattccac gagggcccca gttggtgcc 1020
cagaacttgg gcatcagcct ggccaacttg ttgctgagca aaggagccaa aaacatcctg 1080
gatgttgcac ggcaattgaa cgatgcccat taa 1113

```